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Ultrastructure of Thyroid Gland in Rats Receiving PCBs

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The biological effects of polychlorinated biphenyls (PCBs) has become a matter of concern for environmental toxicologists following the contamination of the global ecosystem by these compounds.

In previous studies we showed the effect of PCBs-1221 on some defense systems of the animal body: The zona fasciculata of the adrenal gland of rats receiving PCBs-1221 showed increased activity (morphological and biochemical). The serum level of gamma-globulin fractions (IgG and IgM) and albumin fell.

This paper reports structural changes in the thyroid gland of rats receiving PCBs. White, local strain, male rats, 4 and 6 weeks old, received 200 and 250 ppm PCBs-1221 in their drinking water for 6 and 10 weeks, respectively. The mean level of PCBs in the adipose tissue of rats receiving 250 ppm PCBs-1221 for 2½ months was 6.8 ppm.

Some thyroid follicular cells of the rats receiving PCBs-1221 showed morphological characteristics of hyperfunction. These cells are tall and contain many colloid droplets. Their basal region is markedly infolded. Other follicular cells presented a vesicular type of rough surfaced endoplasmic reticulum, uncommon to the thyroid follicular cell. In many distended follicles, some of their cells were disintegrated.

In the elucidation of our findings, studies in rats receiving PCBs are currently underway to assess the functional thyroid state and the plasma level of trace elements involved in the activity of thyrocalcitonin secreting cells.

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Absence of Conversion of *o,p'*-DDT to *p,p'*-DDT in the Rat

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Several reports have appeared in the literature which described the in vivo and in vitro conversion of o,p'-DDT to p,p'-DDT (1). These observations were not consistent with experimental results previously obtained at this laboratory, when o,p'-DDT was administered to the rat and squirrel monkey, or with those of Lamont *et al*. (2) when o,p'-DDT was administered to mallard ducks. The conversion of o,p'-DDT to p,p'-DDT is of interest; p,p'-DDT has greater insecticidal activity, o,p'-DDT has been reported to have high estrogenic activity (3), and o,p'-DDD, a metabolite of o,p'-DDT, induces atrophy of the adrenal cortex in man (4) and the dog (5). Additionally, it has been suggested that many of the undesirable effects of technical DDT are due to the o,p'-component (6).

The experiment described in this communication was designed to parallel the studies of Klein *et al*. (1) except that ^{14}C ring-labeled o,p'-DDT (7) was incorporated in the experiment. The purpose of the experiment was to test the report observation that o,p'-DDT is converted to p,p'-DDT by measuring the ^{14}C activity of the p,p'-DDT found in the animals fed ^{14}C-labeled o,p'-DDT. If this conversion does take place, ^{14}C-p,p'-DDT would be recovered.

To study the storage and possible isomeric conversion of o,p'-DDT, 12 female Sprague-Dawley rats weighing 200-250 g each were fed 50 ppm o,p'-DDT in their diet for 12 weeks. No sign of toxicity developed in any animal. For each of the 3 days before the animals were to be sacrificed, ^{14}C-labeled o,p'-DDT (5 μCi, 1 mg) was administered orally in peanut oil to the 12 animals receiving o,p'-DDT. An additional 6 animals were included in the experiment as controls. The animals were sacrificed by decapitation and dissected immediately. Fat samples (5 g) were collected, prepared for residue analysis by the method of Mills, Onley, and Gaither (8), analyzed by gas-liquid chromatography (8), and the results

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[1] Samples were counted with a Nuclear Chicago Mark I liquid scintillation system in vials containing 15 ml of 8g of Butyl PBD and 85 g of naphthalene per liter of p-dioxane.

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confirmed by thin layer chromatography (8). Radioactivity of individual residues was determined by liquid scintillation counting[1] of the individual components trapped in cool capillary tubes attached to the exit of the gas-liquid chromatograph. The absolute recovery of radioactivity was determined by combusting a portion of the fat and determining $^{14}CO_2$. All analytical manipulations were compared to those values obtained by combustion. Recoveries of radioactivity were determined after extraction, as well as after liquid-liquid partition, column, and gas chromatography. Recovery of ^{14}C from the fat was determined to be 85 + 6%. Recoveries of ^{14}C ring-labeled o,p'-DDT and p,p'-DDT, injected into and collected from the gas-liquid chromatograph, were not significantly different and were determined to be 64 ± 11%.

The quantities of residues of p,p'-DDE and p,p'-DDT found in the fat of rats treated with o,p'-DDT were not different from those in the control, as shown in Table 1. The levels of o,p'-DDT in the fat of the animals receiving 50 ppm in their diet as well as ^{14}C-labeled o,p'-DDT were approximately five times those of the animals receiving only ^{14}C-labeled o,p'-DDT. No o,p'-DDE was detected. The lack of change in the storage of p,p'-DDE and p,p'-DDT suggested that o,p'-DDT was not isomerically converted. Increased concentration of o,p'-DDD found in both groups of rats treated with o,p'-DDT indicates that conversion from o,p'-DDT to o,p'-DDD did occur.

TABLE 1

Pesticide residue contents (ppm, mean ± S.E.) of fat from rats fed o,p'-DDT*

Pesticide found	Control (6 rats)	Treatment ^{14}C-o,p'-DDT† (6 rats)	 50 ppm in diet‡ + ^{14}C-o,p'-DDT (12 rats)
p,p'-DDE	1.3±0.19	1.1 ± 0.14	1.2 ± 0.07
p,p'-DDT	3.6±0.48	2.7 ± 0.16	3.3 ± 0.34
o,p'-DDD	N.D.	0.25± 0.03	1.2 ± 0.07
o,p'-DDT	M.D.	2.1 ± 0.31	9.6 ± 0.62

* N.D. = None detectable at 0.1 ppm.
† Given orally for 3 days before sacrifice.
‡ Fed daily for 12 weeks.

The results of the radioassay of these fat samples are shown in Table 2. The lack of any ^{14}C activity in either p,p'-DDT or p,p'-DDE indicates that ^{14}C-o,p'-DDT was not converted to these compounds. The technique used would detect ^{14}C-labeled o,p'-DDT at 20 times back-ground at the level of 0.05 ppm. The finding of radioactivity in the *o*,*p*'-DDD confirms that conversion of *o*,*p*'-DDT to *o*,*p*'-DDD did occur. The consistency of the specific activity of ^{14}C-labeled *o*,*p*'-DDT in the six rats administered only ^{14}C-labeled *o*,*p*'-DDT provided additional confidence in the comparison of results by gas-liquid chromatography and liquid scintillation counting. The nearly equal specific activities of ^{14}C-*o*,*p*'-DDT and ^{14}C-*o*,*p*'-DDD supported the observation that the *o*,*p*'-DDD residue was derived only from the ^{14}C-*o*,*p*'-DDT administered orally. The similarity of ^{14}C concentrations in the fat of rats which had been fed 50 ppm of *o*,*p*'-DDT for 12 weeks + 3 oral doses of ^{14}C-*o*,*p*'-DDT and those receiving only the 3 oral doses suggests that there was no difference in the animals' storage of *o*,*p*'-DDT or *o*,*p*'-DDD due to prior exposure. The low residues of *o*,*p*'-DDT found in this experiment are similar to those reported by Klein *et al*. and reaffirm the suggestion that *o*,*p*'-DDT is more rapidly metabolized than *p*,*p*'-DDT.

TABLE II

Radioassay (mean ± S.E.) of rat fat for residues of *o*,*p*'-DDT, *o*,*p*'-DDD, *p*,*p*'-DDT, *p*,*p*'-DDE (trapped after separation by gas-liquid chromatography)*

		Treatment	
Pesticide found	Control	^{14}C-*o*,*p*'-DDT† (6 rats)	50 ppm in diet‡ ^{14}C-*o*,*p*'-DDT (12 rats)†
p,*p*'-DDE	0	0	0
p,*p*'-DDT	0	0	0
o,*p*'-DDD			
DPM/mg	N.D.	3.0 ± 0.3	3.1 ± 0.4
Specific activity, mCi/mmole	N.D.	1.9 ± 0.2	0.41 ± 0.06
o,*p*'-DDT			
DPM/mg	N.D.	23 ± 2	25 ± 2
Specific activity, mCi/mmole	N.D.	1.8 ± 0.2	0.40 ± 0.05

* N.D. = None detectable; 0 = contained no radioactivity.
† Given orally for 3 days before sacrifice.
‡ Fed daily for 12 weeks.

This work supports the contention that *o*,*p*'-DDT is not converted to *p*,*p*'-DDT in the rat.

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Prenatal Effects of Dieldrin and Photodieldrin in Mice and Rats

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Prenatal Effects of Dieldrin and Photodieldrin in Mice and Rats. CHERNOFF, N., KAVLOCK, R. J., KATHREIN, J. R., DUNN, J. M. AND HASEMAN, J. K. (1975). *Toxicol. Appl. Pharmacol.*, 31, 302–308. Doses of 1.5, 3.0, and 6.0 mg/kg/day of dieldrin and 0.15, 0.30, and 0.60 mg/kg/day of photodieldrin were administered to pregnant CD-1 mice and CD rats by gastric intubation on days 7–16 of gestation. The highest dose of dieldrin induced significant increases in liver/body weight ratios and reduced weight gain in mice, and resulted in 41% mortality in rats. The highest dose of photodieldrin also induced significant increases in mouse liver/body weight ratios, and resulted in 15% mortality in rats. Teratogenic effects were not observed. The higher doses of dieldrin induced an increase in supernumerary ribs and a concomitant decrease in ossification centers in fetal mice indicating toxicity at these dose levels. Similar changes were not found in the rat. Photodieldrin induced no apparent fetal toxicity in either species.

Dieldrin is a highly toxic cyclodiene pesticide. There have been a number of studies concerning its effects on mammalian reproduction, especially in view of its long environmental half-life and its ability to cross the placenta (Eliason and Posner, 1971). Sublethal concentrations of dieldrin administered to mice in the diet for 30 days prior to mating and during gestation produced a significant reduction in litter size (Good and Ware, 1969). Abortions occurred in guinea pigs after administration of 100 ppm dieldrin in the diet, a dose that was also toxic to the mothers (Uzoukwu and Sleight, 1972). Harr *et al.* (1970) showed that dieldrin concentrations above 5–20 ppm in rat fetuses were lethal. They found decreased litter size in animals fed diets in which concentrations of dieldrin reached those values. Diechmann *et al.* (1971) showed a decrease in pup survival in beagle dogs fed aldrin, but their data did not allow for differentiation between a prenatal insult impairing subsequent survival and an effect due to the presence of dieldrin in maternal milk. Boucard *et al.* (1970) reported that dieldrin was teratogenic in rats and mice. The presence of anomalies of doubtful significance and lack of statistical treatment in their paper, however, makes their work difficult to interpret. Ottolenghi *et al.* (1974) found that a single dose of dieldrin administered orally to mice on day 9 or to

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hamsters on days 7, 8, or 9 resulted in open eyes, cleft palate, and webbed feet. The doses used were 30 mg/kg in hamsters, and 15 mg/kg in mice.

Dieldrin may be degraded by sunlight under field conditions (Roburn, 1963; Rosen *et al.*, 1966). The resulting compound, photodieldrin, has been produced in the laboratory and its chemical structure elucidated (Robinson *et al.*, 1966; Burke, 1969). Photodieldrin was found to be more acutely toxic to guinea pigs, rats, and mice (Walton *et al.*, 1971; Walker *et al.*, 1971). No investigations concerning the possible teratogenicity of this compound have been done.

The present study was undertaken to determine the potential teratogenicity and fetotoxicity of dieldrin and photodieldrin administered during the period of organogenesis in two species of mammals, the rat and the mouse.

METHODS

Random-bred albino mice (CD-1 stock) and rats (CD stock) were used.[3] Day 1 of pregnancy was recorded upon demonstration of sperm in the vaginal smear. Mice were housed six to a cage and rats two to a cage in cages with corncob bedding.[4] Animals were kept in constant temperature rooms (22–26°C) with controlled lighting (12 hr of light) and fed commercial lab chow and water ad lib. Approximately 230 animals were used during the course of these experiments.

Dieldrin[5] was administered by gastric intubation at doses of 6.0, 3.0, and 1.5 mg/kg/day during the period of organogenesis on days 7–16 of gestation. Photodieldrin[6] was given during a similar period at doses of 0.60, 0.30, and 0.15 mg/kg/day. Maternal weight on day 6 was used for the calculation of doses. Both compounds were administered as suspensions in peanut oil. Intubation volume was 0.1 ml in all experiments. All mice were treated between 4 and 5 PM and rats between 8 and 9 AM. Peanut oil controls (0.1 ml/day) were used throughout.

Mice and rats were killed on day 18 and 21 of gestation, respectively. The uterus was removed and weighed. Maternal weight differences during gestation were calculated as [(day sacrifice weight–gravid uterus weight)–day 6 weight]. Resorption sites, dead fetuses, and live fetuses were recorded. Live fetuses were weighed and randomly distributed into Bouin's solution (two-thirds of the litter) or 65% isopropyl alcohol (one-third of the litter). All fetuses were examined for external defects. After 3–5 days a necropsy was performed on the fetuses fixed in Bouin's solution. Those in isopropyl alcohol were subsequently cleared in 1% KOH and stained with alizarin red S to facilitate skeletal examination. Sternal and caudal ossification centers were counted as measures of fetal growth retardation (fetotoxicity).

Calculations were based on the litter as the experimental unit and standard deviations, therefore, represent the variation between litter means. Pairwise treatment-control comparisons were made using either the Dunnett test (Miller, 1966) or the Mann–Whitney *U* test (Siegel, 1956) depending upon the distribution of the variable.

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[3] Animals were supplied by the Charles River Breeding Laboratories, Inc.

[4] Deodo grade Sanicel, Paxton Co.

[5] The dieldrin (lot 3041, purity 87%) was obtained from the Perrine Primate Laboratory pesticide repository.

[6] The photodieldrin was synthesized by a method developed by Dr. P. Lombardo at the FDA. Its purity was greater than 95% as checked by gas–liquid chromatography.

The test formulated by Jonckheere (1954) was used to test for an overall dose–response relationship.

RESULTS

Dieldrin: Rat

The data of the rat studies are summarized in Table 1. Rats which received the highest dose (6.0 mg/kg) had a 41 % mortality rate. Deaths generally occurred after the rats had received all 10 doses. Rats which received 6 mg/kg/day gained an average of 55.7 g during gestation which was significantly lower ($p < 0.01$) than the average weight gain of 82.2 g observed in the controls. There were no changes in the liver/body weight ratios of any of the groups.

TABLE 1

PRENATAL EFFECTS OF DIELDRIN IN THE RAT

Observations	Dose group (mg/kg/day)			
	Control	1.5	3.0	6.0
Conception				
No. inseminated	28	14	28	32
No. died	0	0	1	13
No. pregnant (term)	25	9	23	15
Maternal observations				
Av weight gain (g)	82.2 ± 16.7	87.6 ± 22.5	77.1 ± 14.2	55.7 ± 17.5[a]
Av liver/body weight	5.8 ± 0.5	5.9 ± 0.4	5.8 ± 0.5	5.9 ± 0.8
Fetal observations				
Av implants	10.9 ± 2.3	9.7 ± 3.4	10.2 ± 2.4	10.7 ± 2.6
Av mortality (%)	3.1 ± 6.4	3.5 ± 5.3	3.8 ± 6.7	2.1 ± 4.5
Av weight (g)	4.3 ± 0.3	4.8 ± 0.6	4.3 ± 0.3	4.2 ± 0.5
Av no. sternal ossification centers	5.8 ± 0.3	6.0 ± 0.0	5.9 ± 0.2	5.8 ± 0.3
Av no. caudal ossification centers	4.5 ± 0.5	5.0 ± 0.6	4.6 ± 0.7	4.5 ± 0.8
Supernumerary ribs	0	0	1	0

[a] Statistically different from controls, $p < 0.01$.

Fetuses of rats receiving dieldrin showed no significant differences from the controls in mortality, weight, or occurrence of anomalies. There were no significant differences in the average number of sternal or caudal ossification centers.

Dieldrin: Mouse

The mouse studies are summarized in Tables 2 and 3. There was a significant difference ($p < 0.01$) between the average fetal weights on the different sacrifice days, one group averaging 0.94 g and the other 1.28 g. This phenomenon has been noted previously in this strain of mice, both by the senior author and by Courtney and Moore (1971), and is probably related to differences in fetal age. Fetuses in these distinct groups are, therefore, treated separately in the tables and in the statistical calculations.

Mice receiving 6 mg/kg/day gained significantly ($p < 0.05$) less weight when compared to controls. There was a significant ($p < 0.01$) dose-related increase in liver/body weight ratios in the treated groups.

TABLE 2

PRENATAL EFFECTS OF DIELDRIN IN THE MOUSE (EXPT 1)

Data	Dose group (mg/kg/day)			
	Control	1.5	3.0	6.0
Conception				
No. inseminated	14	15	12	15
No. died	0	0	0	0
No. pregnant (term)	8	9	6	7
Maternal observations				
Av weight gain (g)	2.8 ± 2.3	2.5 ± 1.5	3.2 ± 1.3	0.3 ± 2.1[a]
Av liver/body weight	7.3 ± 0.7	8.4 ± 0.6	9.1 ± 1.2[a]	10.7 ± 0.8[a]
Fetal observations				
Av implants	13.1 ± 1.7	12.2 ± 0.8	12.0 ± 2.5	10.9 ± 3.6
Av mortality (%)	18.9 ± 9.7	14.3 ± 12.8	24.5 ± 11.4	8.5 ± 10.2
Av weight (g)	1.28 ± 0.08	1.28 ± 0.10	1.34 ± 0.10	1.21 ± 0.12
Av no. sternal ossification centers	6.0 ± 0.1	6.0 ± 0.0	6.0 ± 0.1	6.0 ± 0.0
Av no. caudal ossification centers	6.2 ± 0.7	6.6 ± 0.8	6.4 ± 1.2	5.0 ± 1.4[a]
Supernumerary ribs (av. litter %)	6.3 ± 17.7	14.8 ± 19.0	41.7 ± 39.1[a]	27.8 ± 22.8[a]

[a] Statistically different from controls, $p < 0.05$.

TABLE 3

PRENATAL EFFECTS OF DIELDRIN IN THE MOUSE (EXPT 2)

Data	Dose group (mg/kg/day)			
	Control	1.5	3.0	6.0
Conception				
No. inseminated	23	20	22	22
No. died	0	0	0	1
No. pregnant (term)	14	15	16	16
Maternal observations				
Av weight gain (g)	3.2 ± 3.1	3.6 ± 2.5	3.6 ± 2.5	2.2 ± 2.4
Av liver/body weight	7.2 ± 0.4	7.6 ± 0.7	8.5 ± 0.9	9.6 ± 1.1
Fetal observations				
Av implants	12.6 ± 2.9	11.9 ± 2.2	12.0 ± 3.1	12.4 ± 1.8
Av mortality (%)	12.6 ± 11.3	19.6 ± 15.3	12.4 ± 12.4	14.8 ± 9.9
Av weight (g)	0.90 ± 0.06	0.98 ± 0.08	1.00 ± 0.14	0.88 ± 0.09
Av no. sternal ossification centers	5.4 ± 0.4	5.0 ± 0.7	5.3 ± 1.3	4.5 ± 1.2
Av no. caudal ossification centers	3.6 ± 0.4	3.0 ± 1.2	3.3 ± 1.0	3.0 ± 0.6[a]
Supernumerary ribs (av litter %)	30.6 ± 35.6	25.0 ± 50.0	19.9 ± 13.5	34.4 ± 40.8

[a] Statistically different from controls, $p < 0.05$.

Fetal skeletal examinations revealed a significantly ($p < 0.05$) increased percentage of supernumerary ribs at the two highest doses in those mice whose fetuses weighed an average of 1.28 g. A significant ($p < 0.05$) decrease in the number of caudal ossification centers was found at the highest dose in these fetuses as well as those weighing an average of 0.94 g. Single examples of cleft palate, imperfect palates, and exencephaly were found, but the treatment–control differences were not statistically significant.

Photodieldrin: Rat

The data of the rat studies are summarized in Table 4. Pregnant rats receiving the highest dose (0.6 mg/kg/day) had a 15% mortality rate. There were no significant differences in fetal mortality, weights, or occurrence of anomalies in litters of treated animals when compared to the controls.

TABLE 4

PRENATAL EFFECTS OF PHOTODIELDRIN IN THE RAT

Data	Dose group (mg/kg/day)			
	Control	0.15	0.30	0.60
Conception				
No. inseminated	31	30	28	34
No. died	0	0	0	5
No. pregnant (term)	27	27	26	24
Maternal observations				
Av weight gain (g)	59.4 ± 18.7	62.4 ± 26.3	56.5 ± 21.8	53.1 ± 19.7
Av liver/body weight	5.5 ± 0.8	5.4 ± 0.6	5.3 ± 0.5	5.4 ± 0.7
Fetal observations				
Av implants	9.7 ± 2.0	9.9 ± 2.8	10.3 ± 2.4	10.0 ± 1.8
Av mortality (%)	6.7 ± 9.2	4.5 ± 7.0	4.3 ± 6.4	8.7 ± 11.4
Av weight (g)	4.7 ± 0.7	4.5 ± 0.5	4.4 ± 0.6	4.4 ± 0.9
Av no. sternal ossification centers	5.9 ± 0.3	5.9 ± 0.3	5.7 ± 0.7	5.6 ± 0.7
Av no. caudal ossification centers	5.3 ± 1.2	5.0 ± 1.1	4.9 ± 1.0	5.0 ± 1.5
Supernumerary ribs	2	3	2	2

Photodieldrin: Mouse

The data of the mouse studies are found in Table 5. A single death (4%) was recorded in the animals which received the high dose (0.6 mg/kg/day). A significant ($p < 0.01$) dose-related increase in liver/body weight ratios was found. The liver/body weight ratios increased from an average of 6.7 in controls to 7.7 at the high dose level.

There were no significant differences in the fetal mortality or weight of litters from animals receiving the different doses. Isolated instances of cleft or defective palate (palates which were asymmetrical or had deep indentations in the midline) were seen in the treated groups, but two defective palates were also noted in control fetuses. Single occurrences of club foot and micrognathia and two fetuses with encephalocoele were also seen in treated groups. There were no significant differences in the percentage of supernumerary ribs, sternal ossification centers, or caudal ossification centers at any of the doses used.

TABLE 5

PRENATAL EFFECTS OF PHOTODIELDRIN IN THE MOUSE

Data	Dose group (mg/kg/day)			
	Control	0.15	0.30	0.60
Conception				
No. inseminated	30	32	32	28
No. died	0	0	0	1
No. pregnant (term)	16	19	20	16
Maternal observations				
Av weight gain (g)	2.4 ± 2.3	2.4 ± 2.4	3.1 ± 3.5	2.8 ± 2.0
Av liver/body weight	6.7 ± 0.7	7.2 ± 0.8	7.3 ± 0.7	7.7 ± 0.7[a]
Fetal observations				
Av implants	13.1 ± 1.9	11.9 ± 2.0	12.2 ± 2.1	10.8 ± 3.2
Av mortality (%)	9.6 ± 7.6	8.3 ± 7.1	13.1 ± 8.9	12.2 ± 10.0
Av weight (g)	1.29 ± 0.10	1.31 ± 0.11	1.32 ± 0.08	1.31 ± 0.11
Av no. sternal ossification centers	6.1 ± 0.2	5.9 ± 0.1	6.1 ± 0.1	6.0 ± 0.1
Av no. caudal ossification centers	6.5 ± 1.1	6.1 ± 1.4	6.7 ± 1.3	6.5 ± 1.2
Supernumerary ribs (av. litter %)	25.2 ± 34.3	18.8 ± 25.3	12.1 ± 27.8	24.6 ± 31.4

[a] Statistically different from controls, $p < 0.05$.

DISCUSSION

Dieldrin was not found to be teratogenic or fetotoxic in the CD rat. A dose which caused 41% mortality and a reduction in the average weight of the surviving mother did not induce any significant differences in the fetuses. Dieldrin was not teratogenic in the CD-1 mouse. The administered doses induced significant liver/body weight ratio increases in the mothers. Fetal toxicity at the highest doses was indicated by decreased numbers of caudal ossification centers and increases in the number of supernumerary ribs.

Photodieldrin was not teratogenic or fetotoxic in either the CD rat or the CD-1 mouse. No significant increases in terata or differences in the average number of ossification centers were noted at any of the doses administered, including those which caused 15% maternal mortality in rats and significant liver/body weight ratio increase in the mouse.

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A Comparative Study of Two Polychlorinated Biphenyl Mixtures (Aroclors 1242 and 1016) Containing 42% Chlorine on Induction of Hepatic Porphyria and Drug Metabolizing Enzymes[1]

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A Comparative Study of Two Polychlorinated Biphenyl Mixtures (Aroclors 1242 and 1016) Containing 42% Chlorine on Induction of Hepatic Porphyria and Drug-Metabolizing Enzymes. GOLDSTEIN, J. A., HICKMAN, P., BURSE, V. W. AND BERGMAN, H. (1975). *Toxicol. Appl. Pharmacol.* 32, 461–473. Aroclor 1242 and Aroclor 1016 are polychlorinated biphenyl (PCB) mixtures with similar chlorine content (42 vs 41%), but Aroclor 1242 contains 9% biphenyl homologs with five or more chlorines while Aroclor 1016 contains only 1%. The effects of Aroclor 1242 and Aroclor 1016 on induction of hepatic porphyria and drug-metabolizing enzymes were compared in female rats fed 100 ppm or 500 ppm of each. At 1 wk, Aroclor 1242 markedly increased liver weight and all drug-metabolizing pathways tested including cytochrome P-450, liver weight, *N*-demethylase, nitroreductase, aniline hydroxylase, and glucuronyl transferase, while Aroclor 1016 had produced only very minimal effects. At 6 mo, however, 500 ppm of either Aroclor markedly increased drug-metabolism, while at the lower dose, Aroclor 1016 was much less effective than Aroclor 1242. Both doses of Aroclor 1242 produced porphyria, but only the higher dose of Aroclor 1016 was porphyrogenic. The porphyria occurred after a lag of 1–6 mo and was characterized by excretion and hepatic storage of uroporphyrins. Aroclor tissue concentrations were similar in rats fed equal doses of the two mixtures. Therefore, the marked differences in the biological effects of Aroclor 1016 and Aroclor 1242 cannot be explained by differences in absorption, metabolism, or excretion.

Polychlorinated biphenyls (PCBs) are industrial chemicals which have been widely used and are widespread contaminants of the environment (Risebrough *et al.*, 1968). In the United States, PCBs are sold under the trademark Aroclor. They are mixtures containing various percentages of organically bound chlorine by weight. PCBs have been found in human tissue and were responsible for an outbreak of chloracne in

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Japan (Yobbs, 1972; Kuratsune *et al.*, 1972). Chronic exposure to PCBs results in porphyria resembling hexachlorobenzene poisoning, characterized by delayed onset and massive increases in urinary and liver uroporphyrins (Goldstein *et al.*, 1974a). PCBs are also known to induce the activity of the hepatic mixed-function oxidase system which metabolizes foreign chemical and endogenous steroids (Litterst *et al.*, 1972). These authors reported that effects on the microsomal mixed function oxidase system correlated directly with the percent chlorine. It has been reported that biphenyls with higher numbers of chlorine per molecule are retained in tissues for longer periods of time, while biphenyls with fewer numbers of chlorines appear to be preferentially metabolized and excreted (Weigel and Smith, 1974; Curley *et al.*, 1971). Preferential retention of highly chlorinated biphenyls could explain the greater biological effectiveness of highly chlorinated PCB mixtures compared with mixtures with lower percent chlorination.

At present, sale of American-made PCBs is limited largely to uses as dielectric fluids in capacitors and transformers. Currently, the major PCB mixture being marketed in the United States is Aroclor 1016, a mixture containing 41.3% chlorine with a very low percentage (1%) of biphenyls with more than four chlorines per molecule. Only one study on the biological effects of Aroclor 1016 has been published. This study compared the effects of Aroclor 1016 with Aroclor 1254 (a biphenyl mixture containing 54% chlorine) on certain pathways of drug metabolism (cytochrome P-450, ethylmorphine *N*-demethylase, aniline hydroxylase, zoxazolamine, and hexobarbital metabolism) after administration to rats of 25 mg/kg/day for 6 days (Bickers *et al.*, 1972). Aroclor 1254 produced up to a 3-fold increase in some drug-metabolizing enzymes, while the maximum increase produced by Aroclor 1016 was 40%. Effects of chronic administration of Aroclor 1016 were not determined. Litterst *et al.* (1972) reported up to threefold increases in nitroreduction of *p*-nitrobenzoic acid and hydroxylation of pentobarbital by Aroclor 1242, a mixture containing approximately 42% chlorine. Because of the reports that, in general, biphenyls with greater numbers of chlorines are excreted, it appeared possible that the lesser effects of Aroclor 1016 could be due to greater metabolism and/or excretion because of its very low content of highly chlorinated biphenyls. In addition, it was not known whether Aroclor 1016 or Aroclor 1242 were less porphyrogenic than other Aroclor mixtures. Therefore, the present study was undertaken to compare the effects of Aroclor 1016 with another chlorinated biphenyl mixture, Aroclor 1242, with similar chlorine content (42%) but containing 9% biphenyls with five or more chlorines vs 1% for Aroclor 1016. Effects of two relatively high doses of Aroclor 1016 and Aroclor 1242 were compared at both 1 wk and 6 mo on a large variety of drug-metabolizing pathways as well as porphyrin accumulation and excretion. In addition, total PCB content of liver and fat were compared after administration of comparable doses of the two compounds.

METHODS

Thirty 1-mo-old female Sherman rats were divided into five groups of six each and fed ground Purina laboratory chow *ad libitum* containing 100 or 500 ppm Aroclor 1016 (lot No. KB-06-756) (Monsanto Chemical Co., St. Louis, Mo.), or plain chow. Rats were sacrificed by decapitation after 1 wk. An additional 30 rats were treated in a similar fashion and sacrificed at 6 mo. Urine was collected in metabolic cages for the 24-hr period preceding sacrifice.

After sacrifice, liver homogenates, 9000 *g* supernates and microsomes were prepared as previously described (Goldstein *et al.*, 1974a). δ-Aminolevulinic acid (ALA) synthetase was assayed in whole liver homogenates (Marver *et al.*, 1966a). *o*-Aminophenol glucuronyl transferase was assayed in microsomes equivalent to 50 mg wet wt of liver (resuspended in 1.15% KCl) in the presence of 0.3% digitonin and 2.7 mol/ml of UDPGA (Goldstein and Taurog, 1968). Aminopyrine *N*-demethylase, aniline hydroxylase, and nitroreductase were assayed in 9000 *g* supernates (Goldstein *et al.*, 1973b). Aniline hydroxylase was assayed in the dark. *N*-demethylase was measured by determination of formaldehyde produced (Cochin and Axelrod, 1959), and aniline hydroxylase by determination of *p*-aminobenzoic acid from *p*-nitrobenzoic acid (Fouts and Brodie, 1957) with the following modification.

Porphyrin in liver fractions from porphyric rats interfered with analysis of products of drug metabolizing enzymes by producing high blanks. In addition, porphyrins interfered specifically with analysis of *p*-aminophenol which is coupled with phenol in alkaline solution to form an indophenol dye and measured spectrophotometrically (Kato and Gillette, 1965). The reaction product was unstable in the presence of porphyric tissue, disappearing variably during the reaction period. Therefore, we routinely removed porphyrin from all protein-free supernatant fractions of drug-metabolizing reactions by the addition of 50 mg/ml talc to the incubation mixtures after the reactions were stopped with acid. The talc, which adsorbed the porphyrins in acid conditions, was precipitated by centrifugation at 2000 *g* for 15 min, and the porphyrin-free supernates were analyzed for *p*-aminophenol, formaldehyde, *p*-aminobenzoic acid, or *o*-aminophenol glucuronide as described above. Recoveries of all compounds were unaffected by talc.

Microsomal cytochrome P-450 and protoheme were determined by the methods of Omura and Sato (1964a, b) using an Aminco DW-2 spectrophotometer. Microsomal protein (Lowry *et al.*, 1951), tissue porphyrins (Doss, 1967), urinary porphyrins (Goldstein *et al.*, 1974a), and urinary ALA and porphobilinogen (PBG) (Marver *et al.*, 1966b) were measured as previously described. Tissue residues of PCBs were analyzed by gas chromatography (GC) using a Micro Tek MT-220 and quantitated by comparing the sum of the peak heights with the sum of total peak heights in the corresponding Aroclor standard (Curley *et al.*, 1971).

Results were analyzed by analysis of variance followed by Duncan's multiple range test (Steel and Torrie, 1960).

RESULTS

At 1 wk, 500 ppm of Aroclor 1242 markedly increased cytochrome P-450 (4-fold), *N*-demethylase (2.5-fold), aniline hydroxylase (6.5-fold), nitroreductase (3-fold), glucuronyl transferase (4-fold), and microsomal protoheme (2.6-fold), and produced moderate increases in ALA synthetase (36%) and liver weight (28%) (Fig. 1). In contrast, 500 ppm Aroclor 1016 produced only a 50% increase in most enzymes. Effects of 500 ppm Aroclor 1016 were significantly lower than those of 500 ppm Aroclor 1242 on all parameters except nitroreductase. The smaller dose of 100 ppm Aroclor 1242 caused significant increases in cytochrome P-450, (2-fold), aniline hydroxylase (2.5-fold), glucuronyl transferase (2-fold), ALA synthetase (22%), microsomal protoheme (60%) and liver weight (9%), while 100 ppm Aroclor 1016 had no effects. The peak of the CO-difference spectrum of cytochrome P-450 was shifted from 450 to 449 nm by Aroclor 1242 treatment but not by Aroclor 1016.

At 6 mo, 500 ppm Aroclor 1242 had lesser effects on cytochrome P-450 and *N*-demethylase than at 1 wk, but aniline hydroxylase, nitroreductase, and liver weight

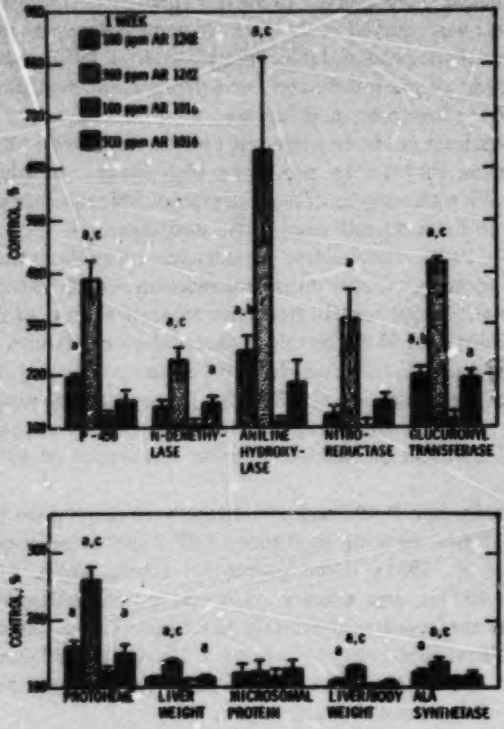

FIG. 1. Effect of Aroclors 1016 and 1242 on cytochrome P-450, drug-metabolizing enzymes, protoheme, liver weight, liver microsomal protein, and ALA synthetase activity at 1 wk. Groups of six female rats were fed 100 or 500 ppm of Aroclors 1016 or 1242 and sacrificed at 1 wk. Values represent means ± SE. a—Significantly greater than controls at $p < 0.05$. b—100 ppm Aroclor 1242 significantly greater than 100 ppm Aroclor 1016, $p < 0.05$. c—500 ppm Aroclor 1242 significantly greater than 500 ppm Aroclor 1016, $p < 0.05$.

remained elevated (Fig. 2 vs Fig. 1). In contrast, the effects of Aroclor 1242 on glucuronyl transferase (13-fold vs 4-fold) and ALA synthetase (5- to 9-fold vs 1.3-fold) were much greater at 6 mo than at 1 wk. In addition, the effects of 500 ppm of Aroclors 1016 and 1242 were roughly comparable at 6 mo; 500 ppm Aroclor 1016 caused marked increases in glucuronyl transferase (11-fold), ALA synthetase (7-fold), nitroreductase (2-fold), aniline hydroxylase (2-fold), and liver weight (29%) (Fig. 2). However, the effects of 500 ppm Aroclor 1016 were significantly lower than the effects of 500 ppm Aroclor 1242 on aniline hydroxylase, nitroreductase, and glucuronyl transferase. At the low dose of 100 ppm, Aroclor 1016 had relatively slight effects, in contrast to the marked effects of Aroclor 1242. These differences were statistically significant for aniline hydroxylase, nitroreductase, glucuronyl transferase, and ALA synthetase.

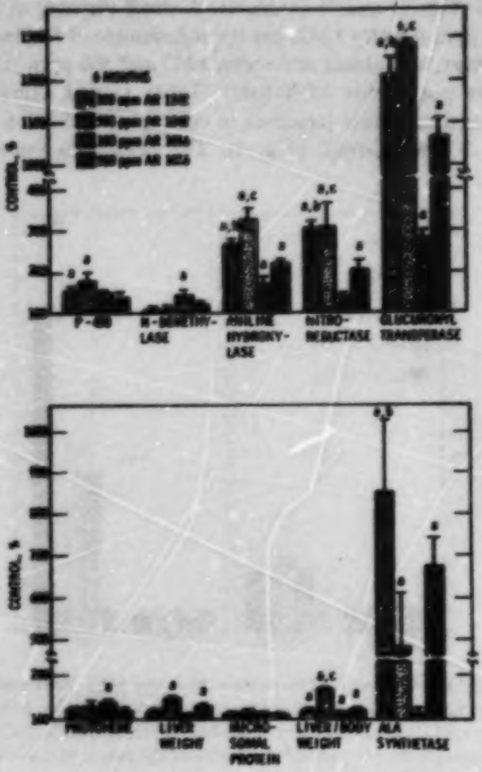

FIG. 2. Effect of Aroclors 1016 and 1242 on cytochrome P-450, drug-metabolizing enzymes, protoheme, liver weight, liver microsomal protein, and ALA synthetase activity at 6 mo. Groups of six female rats fed 100 or 500 ppm of Aroclors 1016 and 1242 were sacrificed at 6 mo. Values represent means ± SE. a—Significantly greater than controls at $p < 0.05$. b—100 ppm Aroclor 1242 significantly greater than 100 ppm Aroclor 1016, $p < 0.05$. c—500 ppm Aroclor 1242 significantly greater than 500 ppm Aroclor 1016, $p < 0.05$.

Weight gain was significantly decreased only by the high dose of Aroclor 1242 at 6 mo (151 ± 11 vs 196 ± 7 g). At 6 mo, the dose of Aroclor consumed was 6 mg/kg/day for rats fed 100 ppm Aroclor 1242 or 1016 and 30 mg/kg/day for rats fed 500 ppm

Aroclor 1242 or 1016. The uterine horns of most rats on both doses of Aroclors 1016 and 1242 were swollen at 1 wk and markedly swollen at 6 mo.

Porphyria

At 1 wk, neither Aroclor 1016 or Aroclor 1242 had any effects on liver or urinary porphyrins or PBG (Figs. 3 and 4) (Table 1). A small increase in urinary ALA was seen in rats fed 500 ppm Aroclor 1242, but the significance of this increase is questionable. At 6 mo, however, both doses of Aroclor 1242 and 500 ppm 1016 produced large increases in hepatic porphyrins (1000-fold) (Table 1) and urinary uroporphyrins (200-fold) (Fig. 4) and moderate increases in coproporphyrin (8-fold), urinary ALA and PBG (approx. 3- and 10-fold) (Fig. 3). The porphyrin found in the livers of PCB-treated rats was identified as 8- and 7-carboxyporphyrins. None of the rats fed 100 ppm Aroclor 1016 showed any evidence of increased liver or urinary porphyrins. Analysis of urinary ALA, PBG, and porphyrins at 12 days, 2 mo, 4 mo, and 6 mo showed that porphyrinuria occurred after 2–6 mo. There was no clear indication of a different time course in the development of porphyria in Aroclor 1242 vs Aroclor 1016 treated rats.

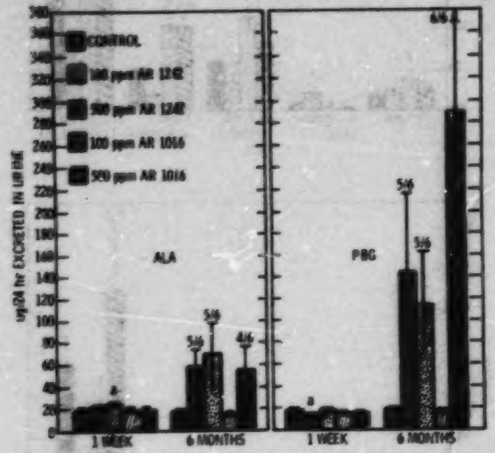

FIG. 3. Effects of Aroclors 1016 and 1242 on urinary ALA and PBG. Rats were treated as described in Figs. 1 and 2, and urine was collected in individual metabolic cages for 24 hr preceding sacrifice. Values represent means ± SE. The numbers above the bars represent the proportion of animals whose individual values were outside the 99% confidence limits of the controls at $p < 0.01$. a—Significantly different from controls, $p < 0.05$.

Tissue PCB Content

Comparing the GC profile of tissue PCBs from Aroclor 1242 and Aroclor 1016 fed animals with the GC profile of the appropriate Aroclor standard, we found disappearance of a number of peaks, changes in the ratios of the peaks, and possible emergence

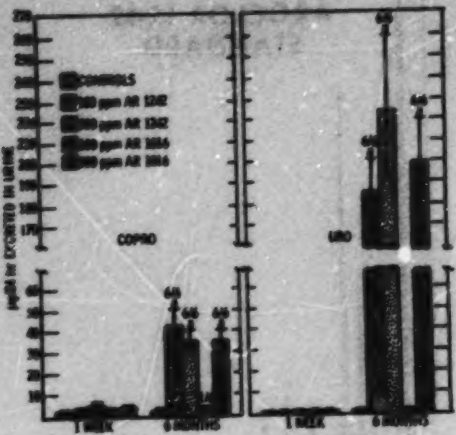

FIG. 4. Effects of Aroclors 1016 and 1242 on urinary coproporphyrin and uroporphyrin excretion. Treatment is described in Fig. 3. Values represent means ± SE. The numbers above the bars represent the proportion of animals whose individual values were outside the 99% confidence limits of the controls at $p < 0.01$. a—Significantly different from controls, $p < 0.01$.

TABLE 1

EFFECT OF AROCLORS 1016 AND 1242 ON LIVER PORPHYRINS IN THE RAT[a]

Treatment	1 Week		6 Months	
	7-COOH	8-COOH	7-COOH	8-COOH
Control	Trace[b]	Trace[b]	Trace[b]	Trace[b]
100 ppm Aroclor 1242	Trace[b]	Trace[b]	164 ± 27	534 ± 61
500 ppm Aroclor 1242	Trace[b]	Trace[b]	227 ± 59	669 ± 146
100 ppm Aroclor 1016	Trace[b]	Trace[b]	Trace[b]	Trace[b]
500 ppm Aroclor 1016	Trace[b]	Trace[b]	89 ± 23	343 ± 65

[a] Porphyrins were methylated and analyzed by thin-layer chromatography. Female rats were treated as described in Fig. 1. Results are given as the mean ± SE.

[b] <1 μg/g.

of additional late-eluting peaks (Figs. 5 and 6). The GC residue profiles of all three tissues from Aroclor 1242 fed rats contained 11 major peaks, while those from Aroclor 1016 fed rats contained five major peaks. The total PCB content was similar in liver and fat of animals fed comparable doses of Aroclor 1242 or Aroclor 1016 (Fig. 7). In particular, rats fed 100 ppm of either Aroclor for 6 mo had comparable liver, blood, and fat PCB concentrations despite markedly different biological effects. At the high dose of 500 ppm, the PCB content of livers from rats fed Aroclor 1242 for 1 wk was significantly higher than liver PCB residues in rats fed Aroclor 1016; however, fat content was comparable.

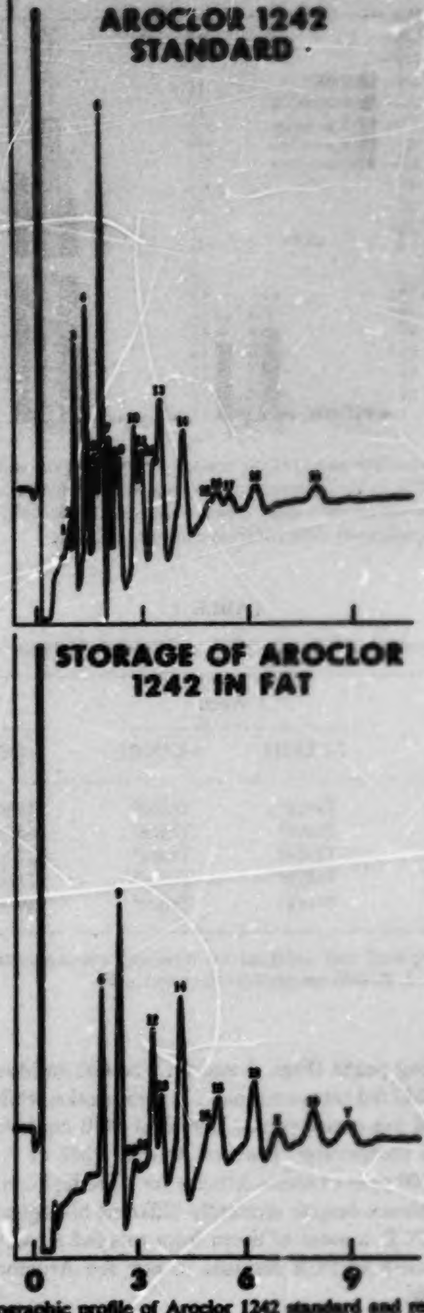

FIG. 5. Gas chromatographic profile of Aroclor 1242 standard and residue from fat of rats fed 100 ppm Aroclor 1242 for 6 mo. Peaks were numbered consecutively according to retention time. Two peaks (labeled *X* and *Y*) were found in extracts of tissues from Aroclor 1242-fed animals but not in the Aroclor 1242 standard.

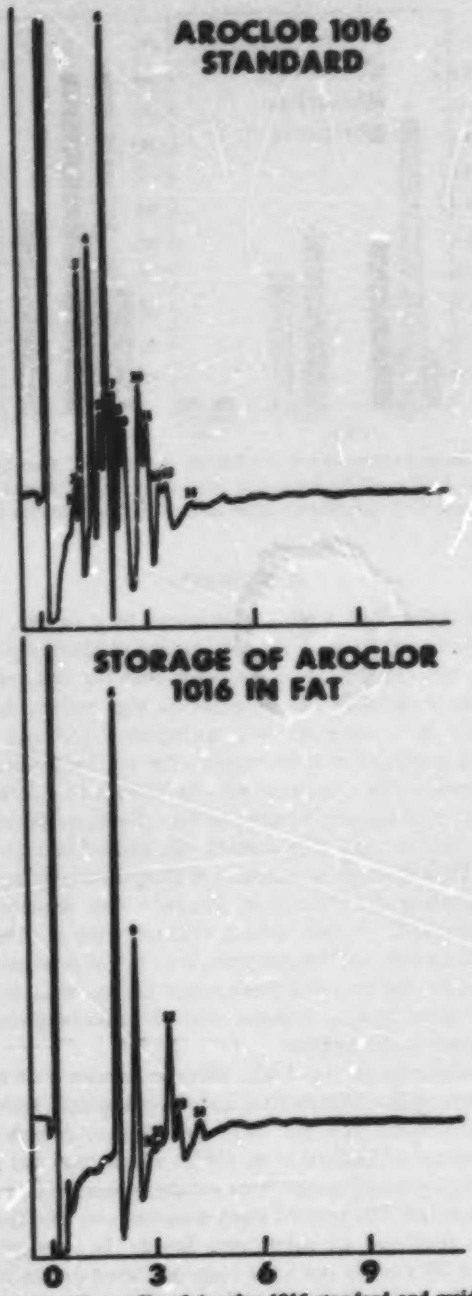

FIG. 6. Gas chromatographic profile of Aroclor 1016 standard and residue from fat of rats fed 100 ppm Aroclor 1016 for 6 mo. Peaks were numbered consecutively according to retention time.

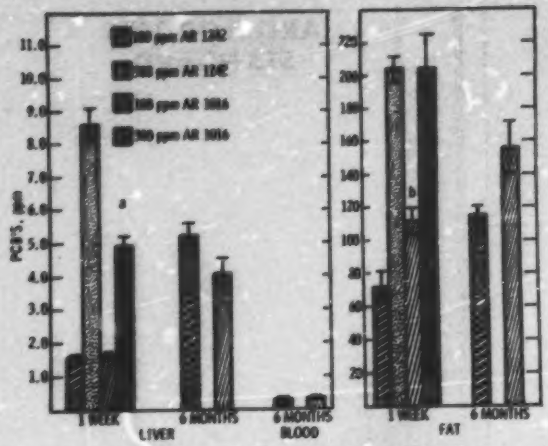

FIG. 7. Tissue PCB content of female rats fed Aroclor 1016 or 1242. Rats were treated as in Fig. 1. Values represent means ± SE. a—500 ppm Aroclor 1016 significantly different from 500 ppm Aroclor 1242. b—100 ppm Aroclor 1016 significantly different from 500 ppm Aroclor 1242.

DISCUSSION

We show that Aroclor 1242 markedly increases liver weight, cytochrome P-450, *N*-demethylase, aniline hydroxylase, nitroreductase, and glucuronyl transferase activity when fed to female rats for 1 wk, while Aroclor 1016 has only very slight effects on liver drug-metabolizing enzymes and no effect on liver weight. Bickers *et al.* (1972) similarly found only 40% increases in cytochrome P-450 and ethylmorphine *N*-demethylase and no increases in liver weight after six daily doses of Aroclor 1016 (25 mg/kg/day). However, he compared Aroclor 1016 with Aroclor 1254, a mixture with higher chlorine content, and concluded that the lesser effects of Aroclor 1016 probably related to the lower chlorine content. Similarly, Litterst *et al.* (1972) showed that the potency of PCB mixtures as inducers of drug-metabolizing enzymes increased with increasing percentage of chlorination. We show that Aroclor 1016 is much less effective than Aroclor 1242, despite similar chlorine content. Therefore, the ability of PCBs to induce drug-metabolizing enzymes does not depend simply on percentage of chlorination. Aroclor 1242 may be a more potent inducer than Aroclor 1016 because it contains a higher concentration of penta- and hexachlorobiphenyls, which may be the most active inducers in the mixture.

In contrast to its minimal effects at 1 wk, 500 ppm Aroclor 1016 markedly increased glucuronyl transferase, aniline hydroxylase and nitroreductase activity as well as liver weight and caused porphyria at 6 mo. These results were completely unexpected in view of the earlier study of Bickers *et al.* (1972) which reported minimal effects of Aroclor 1016 on drug metabolism and liver weight, apparently because they studied only a 6-day dosage period. The present study demonstrates that the effects of chronic exposure cannot be predicted by short-term dosage. In particular, the porphyria produced by Aroclor 1016 could not have been predicted on the basis of short-term studies. The effects of Aroclor 1242 also differed with length of administration. Some effects were less noticeable at 6 mo than at 1 wk; other pathways were increased comparably or even more markedly after long-term administration. We have previously noted that effects of piperonyl butoxide on glucuronyl transferase were much more pronounced after 2 mo, while most other drug-metabolizing enzymes were maximally increased after 1 wk (Goldstein *et al.*, 1973b).

Aroclor 1242 shifted the maximum of the CO-difference spectrum from 450 to 449 nm, but Aroclor 1016 had no discernable effect, possibly because Aroclor 1016 increased cytochrome P-450 content only minimally. A similar shift of the CO-difference spectrum and an increase in the 455/430 ratio of the peaks of the ethyl isocyanide difference spectrum has been noted after Aroclor 1254 administration (Alvares *et al.*, 1973). These changes are similar to those produced by polycyclic hydrocarbons; however, PCBs, unlike polycyclic hydrocarbons, stimulate a variety of pathways.

Both Aroclors 1016 and 1242 produced swelling of the uterine horns. DDT and PCBs with less than 54% chlorination have been reported to have estrogenic effects on the rat uterus (Bitman and Cecil, 1970). Aroclor 1016 appears at least as effective as Aroclor 1242 in the present study. At present, no studies of the effects of Aroclor 1016 on reproduction have been reported.

Previously, we showed that 100 ppm Aroclor 1254 increased urinary excretion and hepatic accumulation of uroporphyrins and induced ALA synthetase, the rate-limiting step in heme synthesis, after a lag of several months (Goldstein *et al.*, 1974a). Both 100 ppm and 500 ppm Aroclor 1242 produced similar effects after 6 mo, while only the high dose of Aroclor 1016 was effective. Apparently, the lag in development of PCB-induced porphyria did not depend on greater tissue accumulation of PCBs with time, since rats fed 100 ppm Aroclor 1242 for 6 mo were porphyric, while rats fed 500 ppm Aroclor 1242 were not porphyric at 1 wk despite higher tissue PCB levels. A similar unexplained lag has been observed in hexachlorobenzene-induced porphyria (Ockner and Schmid, 1961), in contrast to porphyrogenic drugs such as allylisopropylacetamide which increase excretion of porphyrins and their precursors within hours (De Matteis and Prior, 1962).

The lesser effects of Aroclor 1016 than 1242 could not be explained by greater metabolism and excretion, since tissue concentrations were comparable. Other workers in our laboratory found plasma, kidney, brain, liver, and fat content of PCBs were generally higher in rats fed 100 ppm Aroclor 1016 than in rats fed Aroclor 1242 for 0.5, 1, 2, 4, 6, 8, or 10 mo (Burse *et al.*, 1974). It is probable that induction of liver enzymes and hepatic porphyria by PCBs depends on both the number and position of chlorine atoms on the PCB molecule. Significantly, Bush *et al.* (1974) found high correlation between occupation of the 4,4′ positions, tissue retention, cytochrome P-450, and aniline hydroxylase activity after administration of Aroclor 1254. Compounds with one or both *p*-positions open were not persistent in tissue.

Two European commercial preparations of PCBs have been reported to be contaminated with 5–20 ppm of 2,3,7,8-tetrachlorodibenzofuran (TCDF) (Vos *et al.*, 1970). The related compound, 2,3,7,8-tetrachlorodibenzodioxin (TCDD), induces ALA synthetase in the chick embryo at doses as low as 0.5 μg/kg (Poland and Glover, 1973), induces hepatic porphyria in the mouse at doses of 100 μg/kg (Goldstein *et al.*, 1973a), and increases hepatic cytochrome P-450, glucuronyl transferase, and a number of drug-metabolizing enzymes at doses as low as 1 μg/kg (Lucier *et al.*, 1973). Vos *et al.* (1970) could not detect TCDF in American-made PCBs at a sensitivity limit of 1 ppm, but Curley *et al.* (1972) reported contamination of Aroclor 1254 with a trace of TCDF (less than 1 ppm). TCDD was not detected. It is doubtful whether contamination of Aroclors with less than 1 ppm TCDF could attribute appreciably to the hepatic effects of PCBs, since a number of synthetically prepared hexachlorobiphenyls induce ALA synthetase, cytochrome P-450, glucuronyl transferase, and porphyria in the chick, while TCDF does not induce porphyria, even at lethal doses, has little effect on cytochrome P-450, and no effect on glucuronyl transferase or ALA synthetase (Goldstein *et al.*, 1974b). Similarly, TCDF did not induce porphyria in the mouse at 40-times the porphyrogenic dose of TCDD under identical conditions (Goldstein *et al.*, 1974b). Therefore, it is probable that differences in the effects of various PCB mixtures on the liver are due to the effectiveness of the various chlorinated biphenyl homologs in each mixture. Aroclor 1016 is less potent in its effects on the liver, probably because it contains fewer of the biphenyls which are the most active inducers of liver drug-metabolizing enzymes.

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Dehydrogenation: A Previously Unreported Pathway of Lindane Metabolism in Mammals

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This study presents evidence for the dehydrogenation of lindane by a hepatic microsomal mixed-function oxidase system. Preliminary investigation established that the incubation of lindane with rat liver homogenates produces a chlorinated, nonpolar compound identified as hexachlorocyclohexene. Differential centrifugation resulted in the sedimentation of most of the dehydrogenase activity in the microsomal fraction. Optimum *in vitro* assay conditions were established and it was found that the dehydrogenase system required molecular oxygen and reduced pyridine nucleotide coenzyme for maximum activity. Inhibition by SKF 525-A and CO suggested that the enzyme was cytochrome P-450 dependent. Lack of inhibition by cyanide indicated that the cytochrome b_5 desaturase system was probably not involved. Pretreatment of rats with DDT, which stimulates lindane metabolism, also induced significantly higher dehydrogenase activity. Both the *in vivo* and *in vitro* metabolism of hexachlorocyclohexene produced previously identified lindane metabolites. The existence of a cytochrome P-450 dependent mixed-function oxidase which catalyzes the dehydrogenation of lindane has not previously been reported and may be of importance in the metabolism of other xenobiotics.

It is generally accepted that dehydrochlorination of lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane, γ-HCH[1]) to γ-2,3,4,5,6-pentachlorocyclohex-1-ene (γ-PCCH) is the first step in the metabolism of this organochlorine insecticide by both plants (1, 2) and animals (3–14). In 1965 Grover and Sims showed that rats metabolize both lindane and γ-PCCH to 2,3,5 and 2,4,5-trichlorophenol which are excreted in the urine as free phenols, sulfates, and glucuronic acid conjugates (14). Because the metabolites identified in their study resembled those from the degradation of 1,2,4-trichlorobenzene in rabbits (15), the authors speculated that lindane metabolism proceeds via γ-PCCH as an intermediate step to 1,2,4-trichlorobenzene. Currently, most of the published schemes of lindane metabolism are based on this work by Grover and Sims (16–18) and indicate that lindane is metabolized almost exclusively by dehydrochlorination through the intermediate metabolite γ-PCCH. Nevertheless, recent identification of some previously unreported lindane metabolites in flies (11), and the urine of both rats (19) and rabbits (20) led to speculation that there might be important metabolic path-

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[1] The following abbreviations are used: γ-HCH, γ-1,2,3,4,5,6-hexachlorocyclohexane; γ-PCCH, γ-2,-3,4,5,6-pentachlorocyclohex-1-ene; HCCH, Hexachlorocyclohexene; PCCOL, 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol; G6-P, Glucose-6-phosphate; GSH, glutathione; DEGS, diethyleneglycosuccinate.

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ways other than the accepted dehydrochlorination of lindane through γ-PCCH. This study presents evidence to support the existence of a different initial metabolic pathway, specifically the dehydrogenation of lindane to the intermediate metabolite, hexachlorocyclohexene (HCCH).

MATERIAL AND METHODS

Animals

Weanling female Sherman and Sprague–Dawley rats were used in this study. The animals were housed in individual metabolism cages and when treated received po injections of peanut oil solutions of the compounds investigated. Since the rate of lindane metabolism *in vitro* is very low in livers from normal rats, optimum assay conditions were established using liver preparations from rats treated po with 2 mg DDT/day for 2 wk. All animals were sacrificed by decapitation to avoid any complications due to anesthetics.

Chemicals

All pesticides and pesticide metabolites except 1,2-dichlorochlordene, which was provided by Velsicol Chemical Corporation, Chicago, Ill. and those synthesized or isolated from rat urine, were obtained from the Pesticides and Toxic Substances Effects Laboratory, Analytical Reference Standards Repository, Research Triangle Park, N. C. Hexachlorocyclohexene (HCCH) was synthesized by photochlorination of *o*-dichlorobenzene (21). It was isolated by elution with hexane from a silicic acid column. Comparison of synthetic HCCH with the metabolite extracted from liver incubation mixtures, employing lindane as the substrate, demonstrated that the compounds are identical. The mass spectra of the compounds have the same molecular ion, chlorine isotope distribution, and fragmentation pattern. While complete characterization of this intermediate metabolite will be reported in another publication, the salient ions and their intensities include ($M^{\dot{+}}$286 m/e (3%), $M^{\dot{+}} - Cl$ 251 m/e (6%), $M^{\dot{+}} - [Cl + HCl]$ 251 m/e (83%), $M^{\dot{+}} - C_2H_2Cl_2$ 190 m/e (2%), $M^{\dot{+}} - [Cl_2 + HCl]$ 180 m/e (74%)). The compounds have identical retention times on the different glc packing materials used in this study and both elute similarly from a silicic acid column. The lindane metabolite 2,3,4,5,6-pentachloro-2-cyclohexen-1-ol (PCCOL) was isolated from urine and identified previously (19). Dithiothreitol was obtained from Calbiochem, San Diego, Cal. Nicotinamide, glucose-6-phosphate (G6-P), glutathione (GSH), cysteine, G6-P dehydrogenase, NAD$^+$, NADH, NADP$^+$, NADPH, and Trizma preset pH crystals for Tris-HCl buffer were purchased from Sigma Chemical Company, St. Louis, Mo. SKF 525-A was purchased from Smith, Kline, and French Laboratories, Philadelphia, Pa. EDTA was obtained from Fisher Scientific Company, Pittsburgh, Pa. 1.5% OV-17 + 1.95% QF-1, on 80/100 mesh Gas Chrom Q was obtained from Applied Science Laboratories Inc., State College, Pa. Diethyleneglycosuccinate (DEGS) and Gas Chrom Q were obtained from Supelco, Inc., Bellefonte, Pa. Gas chromatographic grade solvents were used for all extractions.

Tissue Preparation

Livers were quickly removed, weighed, and a 1 g portion was homogenized with 5 ml of 0.1 *M* Tris-HCl buffer, pH 7.5, in a Potter–Elvehjem homogenizer with a motor driven Teflon pestle. The homogenization was carried out at 4°C for 1 min. The homogenate was centrifuged at 9000*g* for 20 min in a refrigerated Sorval superspeed centrifuge. The reddish supernatant was used for most of the assays conducted in this study. When microsomes were isolated, the 9000*g* supernatant was centrifuged at 105,000*g* for 75 min in a refrigerated Beckman Model L-2 Ultracentrifuge. The resulting glassy-red pellet was well drained and homogenized by hand into a volume of 0.1 *M* Tris-HCl buffer equal to the volume of the solution that was centrifuged at 105,000*g*. Protein concentration was determined by the Lowry method (22).

Enzyme Assays

The standard incubation mixture of 1 ml of the 9000*g* supernatant (or resuspended microsomes) plus 2 ml of an aqueous mixture containing 160 μmol of Tris-HCl buffer, pH 7.5, 60 μmol of nicotinamide, 2 μmol of NADP⁺, 10 μmol of G6-P, 0.4 units of G6-P dehydrogenase, and 0.34 μmoles of lindane dissolved in 0.1 ml of acetone. The mixture, in a 10 ml Erlenmeyer flask, was incubated for 30 min in a Dubnoff Metabolic Shaking Incubator at 150 oscillations/min and 37°C. The reaction was terminated by the addition of 1.5 ml of 1 *N* HCl to the incubation mixture. In other experiments, acetone solutions of γ-HCCH, cis- and trans-chlordane, and α-, β- and δ-hexachlorocyclohexane were incubated as substrates in place of lindane. In the determination of V_{max} and K_m values, a 20 min incubation time was used.

Extractions

The incubation mixture was transferred to a 15 × 125 mm screwcapped test tube containing approximately 1 cm of NaCl in the bottom of the tube. The incubation flask was rinsed with 1.5 ml of 1 *N* HCl followed by 5 ml of benzene. Both the HCl and the benzene were transferred to the test tube containing the incubation mixture. The combined mixture in the test tube was then shaken vigorously on a vortex mixer for 1 min. The resulting emulsion was broken by centrifugation at 3000 rpm for 3–5 min and the benzene was pipetted off. The extraction was repeated once more and the combined extracts were analyzed by gas chromatography.

Chromatography

Metabolites were assayed on a Micro-Tek MT-220 gas chromatograph equipped with an electron-capture detector and a Coulson electrolytic conductivity detector. The columns used for analysis were 6 ft × 0.25 in. glass U-tubes packed with either 1.5% OV-17 + 1.95% QF-1, on 80/100 mesh Gas Chrom Q or 5% DEGS on 80/100 mesh Gas Chrom Q and both were isothermally maintained at 170°C. The flow of nitrogen carrier gas was maintained at 50 cm³/min.

Statistics

Data were analyzed by the use of Student's *t* test.

RESULTS

In Vitro Metabolism of Lindane in the Rat

Preliminary investigation established that the incubation of lindane with rat liver homogenates produced a chlorinated, nonpolar metabolite which had an identical mass spectra and identical GLC retention time to synthetically prepared hexachlorocyclohexene. The metabolite also eluted from silicic acid similarly to hexachlorocyclohexene. Consequently, the *in vitro* dehydrogenase activities of both control and induced animals were investigated and compared.

Intracellular Distribution

Differential centrifugation resulted in the sedimentation of the highest specific dehydrogenase activity in the microsomal (105,000*g*) pellet as indicated in Table 1.

Optimum Assay Conditions

The 9000*g* supernatant from the centrifugation of rat liver homogenates was used to establish optimum *in vitro* assay conditions for the dehydrogenase system.

Effect of time. Figure 1 indicates that the amount of HCCH formed during the dehydrogenation of lindane was reasonably linear up to 30 min and leveled off thereafter.

TABLE 1

Microsomal Dehydrogenase in Subcellular Fractions Prepared by Differential Centrifugation from Homogenates of Rat Liver

Fraction[a]	Total protein (mg)	Total activity (μg HCCH/ 30 min)	Relative sp act[b]
Homogenate	28	0.299	0.16
Mitochondrial pellet	24	0.069	0.04
9000g Supernatant	23	0.496	0.32
Microsomal pellet	6	0.430	1.00
105,000g Supernatant	17	0.022	0.01

[a] Preparation of subcellular fractions and incubation conditions as described in Methods.

[b] Relative sp act = (Total activity of fraction/ fraction total protein)/(Total activity of Microsomal Pellet/Microsomal Pellet Protein).

Effect of pH. The optimum pH for the dehydrogenase system was 7.5 as indicated in Fig. 2.

Effect of protein concentration. As illustrated in Fig. 3, dehydrogenase activity was proportional to microsomal protein concentration at the level routinely employed in the assay (0.2g wet liver wt or 23 mg of protein in the aliquot of the 9000g supernatant taken for analysis).

Effect of substrate concentration. The apparent Michaelis constants (K_m) for the dehydrogenase systems from both control

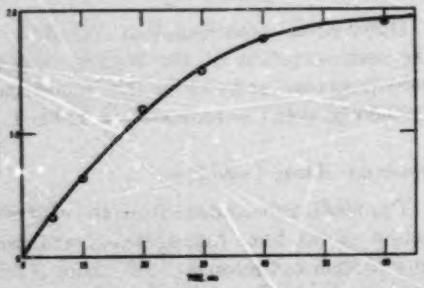

FIG. 1. *Effect of time (minutes) on the* in vitro *metabolism of lindane to hexachlorocyclohexene (μmole/gram liver × 10^{-3}). Incubation conditions as described in Methods. Each value is the mean of two determinations.*

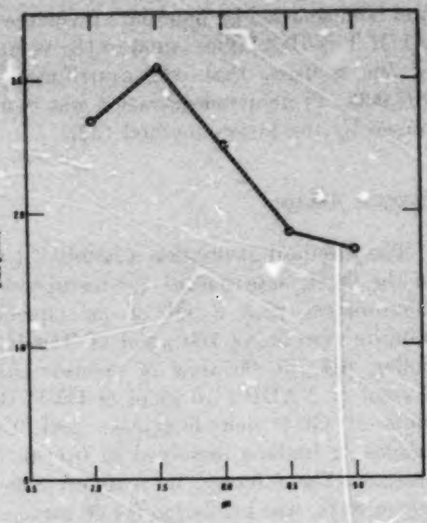

FIG. 2. *Effect of pH on the* in vitro *metabolism of lindane to hexachlorocyclohexene (μmole/gram liver/ hour × 10^{-2}). Incubation conditions as described in Methods. Every value represents the mean of two determinations on each of the liver preparations from two rats.*

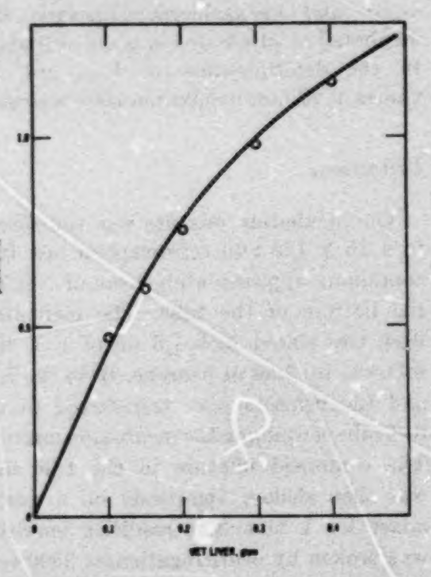

FIG. 3. *Effect of protein concentration (grams wet liver) on the* in vitro *metabolism of lindane to hexachlorocyclohexene (μmole/hour × 10^{-3}). Incubation conditions as described in Methods. Each value is the mean of two determinations.*

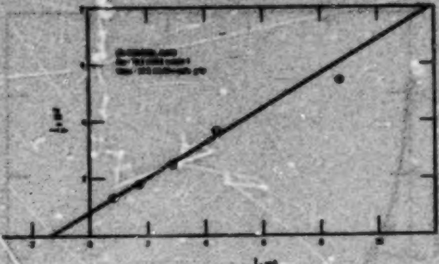

FIG. 4. *Lineweaver–Burk plots for the* in vitro *metabolism of lindane to hexachlorocyclohexene by control rats. S = substrate added (mole/liter); V = product formed (µmole/gram/hour). Incubation conditions as described in Methods. Every value is the mean of two determinations on each of the liver preparations from three rats.*

(Fig. 4) and DDT-treated rats (Fig. 5) were determined from the double reciprocal plots of Lineweaver and Burk (23). The apparent mean K_m ± SE for the DDT and control animals, 20.0 ± 0.60 and 79.2 ± 10.5 µmol of lindane per liter, respectively, were significantly different at $P < 0.01$. The lower K_m value of the DDT treated rats could be the result of a higher affinity between the substrate lindane and the enzyme system, the induction of a new enzyme or perhaps a "deinhibition" of the dehydrogenase enzyme system in these animals. The maximal velocity (V_{max} ± SE) of the control animals (12.5 ± 1.4 nmol HCCH formed/g/h) was increased more than threefold (41.2 ± 10.7 nmol HCCH formed/g/h) by the DDT treatment.

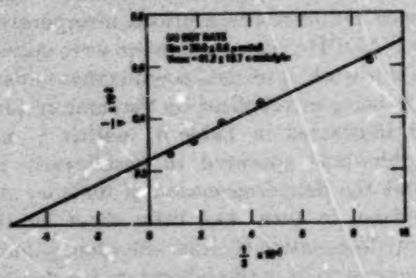

FIG. 5. *Lineweaver–Burk plots for the* in vitro *metabolism of lindane to hexachlorocyclohexene by DDT treated rats. S = substrate added (mole/liter); V = product formed (µmole/gram/hour). Incubation conditions as described in Methods. Every value is the mean of two determinations on each of the liver preparations from five rats.*

TABLE 2

The Coenzyme Requirements of the Dehydrogenase System from Rat Liver

Incubation medium[a]	Relative dehydrogenase activity
Complete 9000g supernatant	100
–dehydrogenase enzymes	0
–dehydrogenase enzymes plus enzymes heated at 100°C, 5 min	0
–Nicotinamide (30 µmol)	62
–Nicotinamide (30 µmol) minus NADPH generating system	6
–NADPH generating system	44
–NADPH generating system plus NADH (2 µmol)	90
–NADPH generating system plus NADPH (2 µmol)	99
–NADPH generating system plus NAD (2 µmol)	78
–NADPH generating system plus NADP (2 µmol)	67
Complete microsomal preparation	100
–NADPH generating system plus NADH (2 µmol)	29
–NADPH generating system plus NADH (10 µmol)	40
–NADPH generating system plus NADH (20 µmol)	48
–NADPH generating system plus NADPH (2 µmol)	45
–NADPH generating system plus NADPH (10 µmol)	115
–NADPH generating system plus NADPH (20 µmol)	118

[a] "Complete" incubation medium and conditions as described in Methods.

Requirements of the dehydrogenase system. As shown in Table 2, the dehydrogenase system required reduced pyridine nucleotide for complete activity and NADPH appeared to be preferred over NADH. While both NADH and NADPH provided almost maximal activity with the 9000*g* supernatant, NADPH supported twofold more activity in the microsomal fraction. The data further suggested that the increased activity provided by NADH in the 9000*g* supernatant was due to the presence of endogenous $NADP^+$ and an NADPH-generating system. Nicotinamide was also required, probably because it protects against NADP degradation (24–29).

Data in Table 3 show that the dehydrogenase system was not affected by addition of 4 mmol EDTA or $MgCl_2$ up to 16 µmol. Cyanide, which is known to inhibit other

TABLE 3

Inhibition of Dehydrogenase Activity

Incubation medium[a]	Relative dehydrogenase activity
Complete 9000g supernatant	100
+Mg Cl_2 (16 μmol)	100
+EDTA (4 mmol)	100
+NaCN (5 μmol)	106
+SKF 525-A (1 mmol)	18
+SKF 525-A (100 μmol)	63
+O_2 (bubbled gently 1 min)	100
+N_2 (bubbled gently 1 min)	0
+CO (bubbled gently 1 min)	0

[a] "Complete" incubation medium and conditions as described in Methods.

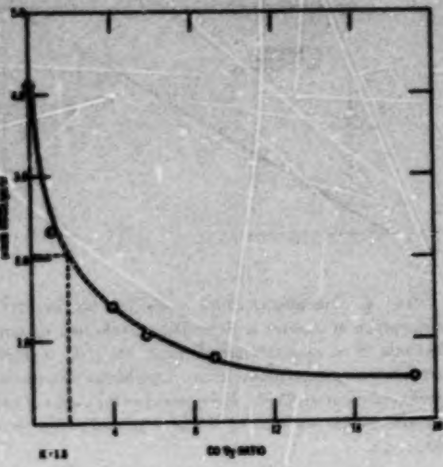

FIG. 6. *Effect of varying the CO/O_2 atmosphere on the* in vitro *metabolism of lindane to hexachlorocyclohexene (μmole/gram liver/hour). The Warburg partition coefficient K = the CO/O_2 ratio required for 50% inhibition of the metabolism of lindane to hexachlorocyclohexene. Incubation conditions as described in Methods. Each value is the mean of two determinations.*

dehydrogenases (30, 31), had little effect on the dehydrogenase system that metabolizes lindane. Though not included in Table 3, glutathione, cysteine, dithiothreitol, and EDTA, which have been used to stabilize or enhance the activity of various dehydrogenases (32–34) had no effect on the dehydrogenation of lindane. Results from Table 3 indicate that SKF 525-A, which by binding to microsomes and soluble cytochrome P-450 greatly inhibits interactions of substrates with cytochrome P-450 (35), was a potent inhibitor of the dehydrogenase activity. Furthermore, dehydrogenase activity was completely inhibited following a brief (1 min) exposure to either CO or N_2. When N_2 was used, lindane was metabolised by a different route to a different metabolite, which was tentatively identified by mass spectroscopy as γ-3,4,5,6-tetrachlorocyclohex-1-ene and the enzyme system appeared to be similar to that reported by Walker (36). Various CO/O_2 ratios were used to determine *K*, the Warburg partition coefficient (37), which is the CO/O_2 ratio required for 50% inhibition of the dehydrogenase system. The ratio was found to be 1.8 (Fig. 6) which is in line with CO/O_2 ratios required for 50% inhibition of various other hepatic microsomal mixed function oxidases (38).

Specificity of the Dehydrogenase System

Both γ-chlordane (1-exo-2-endo-4,5,6,7,-8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene) and α-chlordane (1-exo-2-exo-4,5,6,7,8,8-octachloro-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene) are metabolized *in vitro* by dehydrogenation to the intermediate 1,2,-dichlorochlordene (39). Since rat liver preparations incorporating an NADPH-generating system were utilized in this work, it seemed possible that lindane was being metabolized by the same system. As illustrated in Table 4, neither γ- nor α-chlordane appeared to significantly inhibit the dehydrogenation of lindane, and lindane, in turn, had little effect on the dehydrogenation of either chlordane isomer. Previous work in this laboratory has demonstrated that when separate groups of rats are administered one of four configurational isomers of hexachlorocyclohexane (α-, β, γ-, or δ-HCH) only the group receiving the γ-HCH excreted PCCOL, 2,3,4,6-, and 2,3,4,5-tetrachlorophenol (40). Since these

TABLE 4

Specificity of the Dehydrogenase System from Rat Liver

Substrates utilized[a]	Relative dehydrogenase activity	
	Hexachlorocyclohexene	Dichlorochlordene
γ-HCH	100	
α-chlordane		100
α-chlordane + γHCH	87	89
γ-chlordane		100
γ-chlordane + γHCH	83	94
α-HCH + γHCH	84	
β-HCH + γHCH	88	
δ-HCH + γHCH	54	

[a] One hundred micrograms of each substrate was used in the standard incubation mixture described in Methods.

metabolites could result from a pathway involving a stereospecific dehydrogenase, the metabolism of combinations of lindane (γ-HCH) with the α-, β-, and δ-isomers were compared to that of lindane alone for competitive inhibition of the dehydrogenase system. Only the δ-HCH significantly inhibited the *in vitro* dehydrogenation of lindane indicating that the dehydrogenase exhibited some stereospecificity (Table 4).

In Vivo Metabolism of Hexachlorocyclohexene

Rats administered synthetic HCCH, excreted all previously identified lindane metabolites for which adequate analytical procedures have been developed (Table 5). Moreover, the total metabolism of HCCH to chlorophenols and PCCOL took place almost twice as readily as that of lindane. However, while the quantities of 2,3,5-, 2,4,5-trichlorophenol and 2,3,4,6-tetrachlorophenol excreted by rats receiving HCCH were significantly greater than that excreted by lindane treated rats, the lindane treated animals excreted more 2,4,6-trichlorophenol. No significant differences in the excretion of 2,3,4,5-tetrachlorophenol or PCCOL by the two groups were noted.

In Vitro Metabolism of Hexachlorocyclohexene

The *in vitro* incubation of HCCH with liver preparations produced PCCOL and all five of the chlorophenols recovered from the urine of rats treated with either lindane or hexachlorocyclohexene (Table 6). Pretreatment of the rats with DDT significantly induced the metabolism of HCCH to all of these metabolites. The amount of PCCOL formed was at least five times greater than any of the other metabolites formed *in vitro*, whereas *in vivo* the reverse was true. This could indicate that PCCOL was metabolized further *in vivo* or it could be explained by the fact that optimum incubation conditions were not employed for the other enzymes responsible for HCCH metabolism *in vitro*.

DISCUSSION

Currently, it is accepted that dehydrochlorination of lindane to γ-PCCH is the critical initial step in the metabolism of this organochlorine insecticide by mammals (16–18). However, it is difficult to account for the chemical structure of some previously identified lindane metabolites by a metabolic pathway in which dehydrochlorination is the first step. For example, it was necessary to hypothesize an allylic oxidation with a double bond shift to account for the formation of PCCOL from γ-PCCH in the rat (18), and no attempt has been made to account for the metabolism of lindane to pentachlorophenol in rabbits. Furthermore, a previous report from this laboratory indicated that γ-PCCH is metabolized by the rat to only 2,4,5- plus a trace of 2,3,5-trichlorophenol and not to PCCOL or to the other chlorophenols (40). On the other hand, HCCH is metabolized by the rat to PCCOL and all of the chlorophenols produced from lindane (Tables 5

TABLE 5

Comparative in vivo Metabolism of Lindane and HCCH

Compound administered[a]	Average μg metabolite excreted/24 H ± SE[b]					
	246-TCP[c]	235-TCP[d]	245-TCP[e]	2346-TTCP[f]	2345-TTCP[g]	PCCOL[h]
Lindane	51.1 ± 8.27	8.43 ± 0.961	14.7 ± 3.22	27.1 ± 4.06	4.32 ± 2.82	0.25 ± 0.067
HCCH	20.6 ± 4.10[i]	33.6 ± 2.50[i]	50.1 ± 4.24[i]	86.8 ± 11.2[i]	5.39 ± 0.990	0.48 ± 0.120

[a] Two milligrams of the respective compound/0.1 ml peanut oil was administered to each rat.

[b] Excretion values represent the mean of six animals ± SE.

[c] 2,4,6-Trichlorophenol.

[d] 2,3,5-Trichlorophenol.

[e] 2,4,5-Trichlorophenol.

[f] 2,3,4,6-Tetrachlorophenol.

[g] 2,3,4,5-Tetrachlorophenol.

[h] 2,3,4,5,6-Pentachloro-2-cyclohexen-1-ol.

[i] $P < 0.05$.

and 6). A pathway involving the initial dehydrogenation of lindane to HCCH should significantly simplify attempts to account for known lindane metabolites in mammals.

Apparently, this is the first report of a cytochrome P-450 dependent mixed function oxidase which requires molecular oxygen and reduced pyridine nucleotide coenzyme, and catalyzes the dehydrogenation of a pesticide. Although other microsomal dehydrogenases have been reported (41–46), these do not require cytochrome P-450 or reduced pyridine nucleotide coenzyme. Though both systems require molecular oxygen and reduced pyridine nucleotide coenzyme, the mixed-function oxidase responsible for lindane dehydrogenation differs from the fatty acid desaturase described by Oshino (31) in that the dehydrogenation of lindane is cytochrome P-450 dependent and not sensitive to cyanide inhibition, whereas the opposite holds true for the desaturase system.

It is possible that the dehydrogenase system metabolizing lindane is the same

TABLE 6

In Vitro Metabolism of Hexachlorocyclohexene by Rat Liver[a]

Animal pretreatment[b]	Average μg metabolite produced/30 min					
	246-TCP[c]	235-TCP[d]	245-TCP[e]	2346-TTCP[f]	2345-TTCP[g]	PCCOL[h]
Control	2.2 ± 0.12	0.74 ± 0.10	1.3 ± 0.07	4.3 ± 0.40	0.50 ± 0.06	25 ± 3.5
DDT	4.8 ± 0.05[i]	2.1 ± 0.18[i]	6.0 ± 0.23[i]	11 ± 0.36[i]	0.92 ± 0.04[i]	40 ± 2.9[i]

[a] Four hundred micrograms of HCCH was used in the standard incubation mixture described in Methods.

[b] Pretreatment as described in Methods.

[c] 2,4,6-Trichlorophenol.

[d] 2,3,5-Trichlorophenol.

[e] 2,4,5-Trichlorophenol.

[f] 2,3,4,6-Tetrachlorophenol.

[g] 2,3,4,5-Tetrachlorophenol.

[h] 2,3,4,5,6-Pentachloro-2-cyclohexen-1-ol.

[i] $P < 0.05$.

as that which converts α- and γ-chlordane to 1,2-dichlorochlordene (39) or that which dehydrogenates pyrrolizidine alkaloids (47). However, the intracellular distribution and cofactor requirements of the enzyme which dehydrogenates chlordane have not been reported. Furthermore, saturating levels of either α- or γ-chlordane fail to significantly inhibit the dehydrogenation of lindane *in vitro*, and lindane has little effect on the dehydrogenation of either chlordane isomer (Table 4). This suggests that either different dehydrogenases or different binding proteins for the same dehydrogenase may be involved here. Similarities between the microsomal enzyme catalyzing the dehydrogenation of lindane and that reported to convert pyrrolizidine alkaloids to dihydropyrrolizine derivatives include a requirement for molecular oxygen, reduced NADP+, and inhibition by CO and SKF 525-A together with induction by DDT pretreatment (47). The authors of that study indicate that a new type of microsomal reaction need not be hypothesized. They propose a mechanism for the dehydrogenation of pyrrolizidine alkaloids which might involve initial C-hydroxylation or N-demethylation followed by a spontaneous sequence of nonenzymatic reactions. Such a mechanism, however, is not applicable to the metabolism of lindane. Moreover, the fact that the dehydrogenation of lindane apparently does involve a previously unreported type of microsomal reaction leads to speculation that the pyrrolizidine alkaloids and lindane may actually be metabolized by the same enzyme system. The metabolic dehydrogenation of these alkaloids produces toxic pyrrole derivatives and the detoxification of at least two pesticides, lindane and chlordane, may involve the same pathway. This enzyme system should be further characterized to determine its possible implication in the toxicology and metabolism of other xenobiotics.

Hamilton has suggested that the oxenoid mechanism may be involved in the dehydrogenation catalyzed by microsomal enzymes (48). On the basis of analogies with carbene (49) and nitrene (50) reactions, in addition to model reaction studies, Hamilton proposed that the equivalent of a free oxygen atom, with its six outershell electrons, abstracts two hydrogens to give water and the alkene directly (Fig. 7).

The oxenoid reagent E − X = O is generated from O_2 and a group on the enzyme by reduction. Hamilton has speculated that the structure of the oxenoid reagent could be a peracid or a peramide formed from a carboxyl or amide group and the superoxide ion $O_2^{-}\cdot$, or Fe (III)·$O_2^{-}\cdot$. In numerous examples of the oxidative formation of nonactivated double bonds (conjugated at the most to a second double bond), the removal of two hydrogens occurs by a *cis* elimination and is consistent with the oxenoid mechanism (51–62). This list includes double bond formation in steroid rings, steroid side chains, and fatty acids.

It is proposed that the dehydrogenation of lindane by a hepatic, cytochrome P-450 dependent mixed function oxidase represents an important initial step in the metabolic pathway leading to the detoxification of this pesticide by mammals.

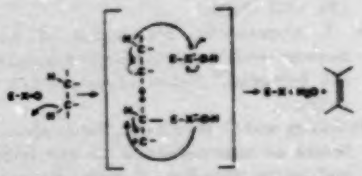

FIG. 7. *Possible mechanism for the dehydrogenation of lindane by microsomal cyt. P-450. E − X = O represents the oxenoid reagent, generated from O_2 and a group on the enzyme, which abstracts two hydrogens from a substrate such as lindane to give water and the alkene directly.*

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Biochemical Effects of Repeated Administration of *p,p′*-DDT on the Squirrel Monkey

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Biochemical Effects of Repeated Administration of *p,p′*-DDT on the Squirrel Monkey. CRANMER, M., PEOPLES, A., AND CHADWICK, R. (1972). *Toxicol. Appl. Pharmacol.* 21, 98–101. *p,p′*-DDT was administered po in peanut oil to 5 groups of 6 squirrel monkeys at doses of 50, 5, 0.5, 0.05, and 0 mg/kg/day. Blood samples were taken weekly from each animal; several hematologic parameters were determined, and plasma amylase, aldolase, glutamic-pyruvic transaminase, and isocitric dehydrogenase were measured. Two monkeys from each group were killed after 2, 4, and 6 mo and in vitro liver homogenate enzyme activity was measured using *p*-nitroanisole and ethyl *p*-nitrophenyl phenylphosphonothioate as substrates. No discernible effects on hematologic values or plasma enzyme levels were observed. The stimulation of the 2 liver homogenate mixed-function oxidases was maximal after 2 mo treatment. There were no significant differences in the levels of homogenate enzyme activity at the 3 sacrifice periods. The monkeys given 50 mg/kg/day all died within 14 wk of starting treatment; deaths did not occur at the other doses.

Primates are finding increasingly wider use in biomedical research because in certain instances man has a closer physiological resemblance to primates than to other more common laboratory animals (Smith, 1966). They are especially useful for assessing the effects of long-range human exposure to toxic materials.

The purpose of this study was to observe the physiological changes in the squirrel monkey after exposure to 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (*p,p′*-DDT). The parameters examined were chosen as those most likely to yield data that could be compared to those obtained from exposed humans.

METHODS

Squirrel monkeys (*Saimuri sciureus*) were purchased as acclimated monkeys[1] and had been imported from Iquitos, Peru. The group consisted of 18 female and 12 male adult monkeys weighing between 500 and 1000 g. The animals were isolated in individual metabolism cages at the Perrine Primate Laboratory for 60 days before the preexposure tests. Room temperature was maintained between 72 and 74°F with a lighting period from 8 AM to 4:30 PM. The monkeys were fed Purina monkey chow (25% protein) ad libitum, and each was given 1/4 apple per day. Water was provided from water bottles.

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[1] Safari Animal Imports, Miami, Florida.

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For 3 mo before treatment with *p,p′*-DDT was begun, baseline measurements were obtained weekly for each animal.

The experimental treatment period was designed to yield in vivo and in vitro data related both to time and dose. The 18 female and 12 male squirrel monkeys were randomly separated into 5 groups of 6 animals without regard to size or sex. *p,p′*-DDT in peanut oil was administered po with a dosing needle to 4 groups of monkeys at doses of 50, 5, 0.5, or 0.05 mg/kg/day. The fifth group served as controls and was given peanut oil without the insecticide. The exposure decreased over a range which included levels producing lethal effects and levels representing the exposure of the human population. It was expected that some of those monkeys given 50 mg of *p,p′*-DDT per kilogram of body weight per day would survive at least a few weeks and perhaps the entire duration of the experiment. Fatalities were not expected at the other doses.

Blood samples were collected weekly in the mornings by lancing the saphenous vein and taking samples from the freely flowing blood. Erythrocytes and white cells were diluted with Hayem solution and 2% acetic acid, respectively, and counted with a hemacytometer; blood smears were prepared with Wright stain; and hemoglobin was determined spectrophotometrically using cyanmethemoglobin reagent (Davidsohn and Wells, 1962). Total protein was determined with a Goldberg hand refractometer on plasma from a hematocrit tube (Barry *et al.*, 1960).

Blood samples for enzyme determinations were obtained by venipuncture using vacuum tubes containing heparin. The plasma was separated by centrifugation and immediately analyzed for enzyme activity. The enzymes studied were aldolase, amylase, glutamic-pyruvic transaminase (GPT), and isocitric dehydrogenase (ICD).[2]

Liver Microsomal Enzymes. At the end of 2, 4, and 6 mo of treatment, 2 monkeys from each group were sacrificed by decapitation. Livers were removed, weighed, and held in an ice bath until determination of the in vitro liver homogenate enzyme activity using *p*-nitroanisole (PNA) and ethyl *p*-nitrophenyl phenylphosphonothioate (EPN) as substrates (Kinoshita *et al.*, 1966). Because all monkeys given *p,p′*-DDT at mg/kg/day died, no liver homogenate enzyme activity data could be obtained for this group. The data were analyzed by use of the multiple range and *F* tests (Duncan, 1955).

RESULTS

Liver Homogenate Enzymes

The multiple range and *F* tests indicated that, in the group given DDT at 5 mg/kg, hepatic homogenate enzymatic hydrolysis of EPN was significantly different from that of the groups receiving 0.5 mg/kg or 0.05 mg/kg and from the control group (Table 1). No significant differences due to duration of treatment were noted. These data parallel the findings of Kinoshita *et al.* (1966) that after the initial induction of EPN detoxification, activity was maintained at a constant level.

In the group given DDT at 5 mg/kg, hepatic homogenate *O*-demethylase activity was significantly different from that of the groups given 0.5 or 0.05 mg/kg and from the control group (Table 1). In addition, *O*-demethylase activity of the group given 0.5 mg/kg was significantly different from that of the group given 0.05 mg/kg and from the control group. The duration of treatment did not affect the activity.

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[2] Sigma Technical Bulletin No. 750—aldolase; Bulletin 700—amylase; Bulletin 505—GPT; Bulletin 157—ICD: Sigma Chemical Company, St. Louis, Missouri.

TABLE 1

EFFECT OF *p,p′*-DDT ON HEPATIC MICROSOMAL ENZYME ACTIVITY OF SQUIRREL MONKEYS

	μg EPN hydrolyzed/50 mg liver[a]			μg PNA hydrolyzed/50 mg liver[a]		
p,p′-DDT (mg/kg/day)	2 Mo	4 Mo	6 Mo	2 Mo	4 Mo	6 Mo
Control	6, 7	6, 7	6, 6	13, 16	14, 15	8, 12
0.05	7, 9	5, 5	4, 8	14, 17	9, 16	10, 15
0.5	6, 8	4, 5	4, 6	28, 34[b]	26, 32[b]	28, 30[b]
5.0	14, 18[c]	15, 19[c]	15, 17[c]	37, 48[c]	45, 58[c]	50, 62[c]

[a] Each figure represents the average of duplicate results. For EPN the duplicates varied 0 to 6.6% from the average and the p-NA determination varied 0 to 11.1% from the average.

[b] Significantly more activity than control and 0.5 mg/kg group ($p < 0.05$).

[c] Significantly more activity than all other groups ($p < 0.05$).

Hematology and Plasma Enzyme Activity

The measurement of hematologic parameters and plasma enzyme activity yielded little information useful for assessing the effects of *p,p′*-DDT on the squirrel monkey. Values were not significantly changed during the treatment period for any of the animals as compared to the preexposure baseline values (Peoples *et al.*, 1969).

The broad range of enzyme values at the basal level indicated that statistical significance might not be observed, and this proved to be true. This laboratory felt that the excitability of, and the stress on, the squirrel monkey in regard to blood taking might be responsible in part for the wide range of values observed. With this in mind the following technique was used when blood was drawn. Each monkey was bled twice a week. If the time the attendant approached the cage door until the blood was drawn was greater than 2 min, the monkey was put back in the cage, and no further attempt was made until the following day.

Toxicologic Observations

All the animals given *p,p′*-DDT at 50 mg/kg/day died during the first 14 wk of the experiment. These monkeys showed initial clinical signs of toxicity (staggering, weight loss, weakness, and loss of equilibrium); all became so weak that they could not remain upright. However, the monkeys began to recover without additional attention, although the administration of *p,p′*-DDT was continued; they regained strength and weight and lost all obvious signs of *p,p′*-DDT toxicity. Then, after 4 wk of treatment, 4 of the monkeys died unexpectedly without showing any of the earlier signs of toxicity. The remaining 2 monkeys of this group succumbed during the 9th and 14th wk of treatment.

DISCUSSION

There were no discernible effects due to treatment with *p,p′*-DDT on the hematology or on the plasma aldolase, amylase, or isocitric dehydrogenase in the squirrel monkey.

The 2 mixed-function oxidases from liver homogenates were significantly stimulated after 2 mo treatment with *p,p′*-DDT. However, the enhancement of the activity of the system which is responsible for the oxidation and hydrolysis of EPN was observed only at the *p,p′*-DDT dose of 5 mg/kg/day, suggesting that the effect level of *p,p′*-DDT required to stimulate the enzymatic system responsible for EPN degradation is greater than the effect level for *O*-demethylase induction (0.5 mg/kg/day). The observation that liver homogenate enzymes were induced at treatment levels which produced no signs of toxicity or alteration in plasma enzymes suggests that hepatic homogenate induction may be an adaptive process (Hoffman *et al.*, 1970). There were no significant differences for levels of activity of either enzyme among the 3 sacrifice periods, indicating that induction was completely stabilized prior to the 8th wk of treatment.

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PB 280 896

Oxychlordane Residues in Human Adipose Tissue

PESTICIDE PUBLICATION
645

by

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Recent investigations have demonstrated that the octachloro isomers of the insecticide chlordane are oxidatively metabolized in mammalian systems to oxychlordane (1-exo-2-endo-4,5,6,7,8,8-octachloro-2,3-exo-epoxy-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene), probably via a 1,2-dichlorochlordene-2 intermediate (SCHWEMMER, et al., 1970 and STREET, et al., 1971). Residue storage levels of this non-polar, lipid soluble metabolite have been determined in several species including rats, dogs, swine, and cattle under controlled feeding conditions (POLEN, et al., 1970). In addition, residues of oxychlordane have been found in milk from cows which had been feeding on alfalfa contaminated with chlordane (LAWRENCE, et al., 1970). In this communication, we wish to report the confirmation and consistent finding of oxychlordane residues in general population human adipose tissue samples obtained through the National Human Monitoring Program (YOBS, 1971) for pesticides administered by the Office of Pesticides, U. S. Environmental Protection Agency.

Adipose tissue specimens are routinely collected from post-mortem examinations and therapeutic surgery procedures and analyzed for a series of organochlorine pesticide residues using a modified Mills-Onley-Gaither procedure (MILLS, et al., 1963 and THOMPSON, 1971). The results of a typical electron capture gas chromatographic determination of a purified tissue extract (Florisil column chromatography, fraction 1 eluate) illustrating the elution characteristics of oxychlordane are presented in Figure 1.

Analytical conditions are also indicated. A representative series of twenty-seven tissue specimens were analyzed and oxychlordane residue determined. Mean values, standard deviations and ranges for this group are presented in Table 1.

TABLE 1

Oxychlordane Residues in General Population Human Adipose Tissue Samples

Mean and Std. Deviations (ppm)	No. of Specimens	Range (ppm)	% Specimens Positive
0.14 ± 0.09	27	0.03 - 0.40	21/27 (77.8%)

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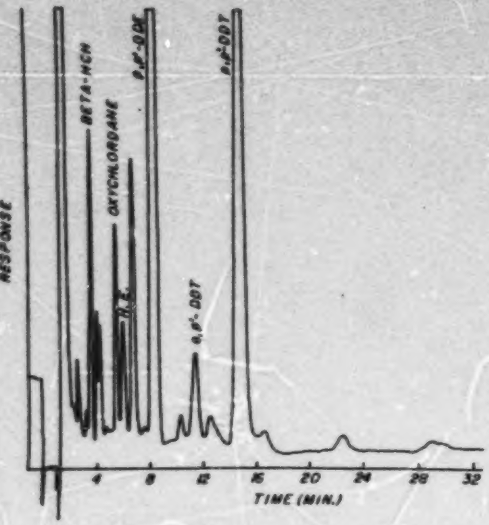

Figure 1. Organochlorine pesticide residues, including oxychlordane, in human adipose tissue extract. Electron capture gas chromatographic determination on column of 1.5% OV-17/1.95% QF-1 on 80/100 mesh silanized support at 200°C oven temperature and nitrogen carrier flow of 60 ml/min.

Routine monitoring laboratory procedure provided for the confirmation of the organochlorine pesticide residues including oxychlordane by comparison of qualitative and quantitative results employing two gas chromatographic columns (1.5% OV-17/1.95% QF-1, RRT_{aldrin} of oxychlordane, 1.38 at 200°C; 5% OV-210 RRT_{aldrin} of oxychlordane, 1.56 at 180°C) and thin layer chromatography. In this instance, additional instrumental confirmation and characterization of the oxychlordane was obtained using infrared spectroscopy and mass spectrometry. Quantities of residue sufficient for confirmation by these techniques were obtained using the following procedure. Human adipose tissue (500 g total) was extracted in the usual manner (THOMPSON, 1971) with petroleum ether. The extract was filtered, partitioned with acetonitrile, dried, concentrated, and fractionated and purified by Florisil column chromatography. The eluate from the first fraction (6% diethyl ether in petroleum ether eluate, Figure 1), containing the oxychlordane as well as remaining organochlorine pesticides except dieldrin, was concentrated and additionally purified by chromatography through a second Florisil column employing 200 ml petroleum ether (fraction 1) and 250 ml 1% (v/v) methanol in petroleum ether (fraction 2) as eluates. The second eluate containing oxychlordane and the more polar polycyclic chlorinated pesticides including the hexachlorocyclohexane isomers, was concentrated and finally fractionated and purified by silica gel column chromatography to isolate the chlordane metabolite residue. Elution with 15% (v/v) benzene in hexane provided material sufficiently free of interfering residues and tissue coextractives for infrared analysis. For mass spectral analysis, however, a sample of oxychlordane was obtained by gas chromatographic trapping of the material in this eluate.

The infrared spectrum of the isolated residue compared well with that obtained for an authentic sample (FDA analytical reference standard) and available literature data (SCHWEMMER, et al., 1970, POLEN, et al., 1970, and LAWRENCE, et al., 1970). The following observed frequencies of absorption (cm^{-1}) were considered characteristic: 1605, fully substituted olefinic bond; 1240, 1250, epoxide C-O bond stretch; 823, 897, 910, epoxide ring vibrations; and finally, 690, 713, C-Cl bond stretching.

Mass spectral analysis at 80 EV of the oxychlordane residue employing direct probe introduction techniques revealed a relatively weak molecular ion of m/e 420. Further fragmentation of the parent molecule resulted in major daughter ions of m/e 385, formed by loss of a single chlorine atom (seven chlorine atom isotope intensity distribution); m/e 235, 270, retro Diels-Alder fragments of probable $C_5Cl_5^+$ -and $C_5Cl_6^+$ composition, respectively; m/e 149, 115 (base peak) fragments corresponding to the cyclopentane oxide moiety of the parent molecule formed by retrodiene decomposition with possible $C_5H_3Cl_2O^+$ and $C_5H_4ClO^+$ composition; and finally, a major isotope group of m/e 183 corresponding to a fragment species containing three chlorine atoms (SCHWEMMER, et al., 1970, and LAWRENCE, et al., 1970).

The occurrence of storage of oxychlordane residues in general population human adipose tissue may indicate consistent previous exposure to chlordane insecticide and/or oxychlordane from many possible sources. With the inclusion of this pesticide metabolite in the human tissue residue monitoring program, significant trends and possible sources of exposure, as well as potential health effects may be assessed more accurately. Human toxicity, dose storage and response relationships, extent and distribution in humans, long term low level exposure effects and other factors including the determination of oxychlordane as a human metabolite of chlordane remain to be determined.

ACKNOWLEDGEMENTS

We thank Dr. A. R. Yobs, Division of Pesticide Community Studies, Office of Pesticides, U. S. Environmental Protection Agency for providing the human adipose tissue samples for study; Mr. J. F. Thompson for data concerning the analytical behavior of oxychlordane; and Miss A. Medbery for excellent technical assistance.

Note added in proof: Independent studies performed by Food and Drug Administration laboratories (BURKE and McMAHON) have also confirmed oxychlordane residues in human adipose tissue. Identification in this instance has been supported by gas and thin layer chromatographic data.

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The Ultrastructure of Livers of Rats Fed DDT and Dieldrin

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PESTICIDE
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Adult male rats were fed different [illegible] of DDT, dieldrin, or combination of the [illegible] eight weeks, hexobarbital sleeping time was determined in some of these rats. Livers of the other rats were examined with the light microscope and electron microscope. Hexobarbital sleeping time was significantly reduced in all treated rats compared to that of controls, but no difference was found between the various treated groups. Liver weights of all treated groups were significantly higher than those of the controls, and livers of rats fed the combination of DDT and dieldrin weighed significantly more than those rats given DDT or dieldrin alone. Morphological changes, including an increase in smooth endoplasmic reticulum and atypical mitochondria, were observed in all treated groups and were more pronounced in rats given both DDT and dieldrin.

BOTH dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-*endo-exo*-5,8-dimethanonaphthalene) and DDT [1,1,1-trichloro-2, 2-bis(*p*-chlorophenyl) ethane], if ingested in sufficient quantity over a period of time, lead to enlargement of the liver in rats and dogs.[1-4] Both compounds stimulate microsomal enzymes and increase hepatic smooth endoplasmic reticulum.[5-8]

Since concomitant environmental exposure to DDT and dieldrin can occur, and since there are indications that dieldrin metabolism is affected by DDT,[9,10] the effect of DDT and dieldrin alone and in combination on the ultrastructure of the rat liver was studied. The hexobarbital sleeping time of rats treated with these compounds was also determined. The results of these studies are reported in this paper.

Materials and Methods

Ninety Sherman strain male rats, 3 to 4 months old and weighing 342 to 512 gm, were distributed according to a table of random numbers into six groups of 15 rats each. The rats were individually caged and weighed each week.

DDT and dieldrin were technical grade and were fed as a component of the diet. Dietary levels fed to the respective groups of rats are presented in Table 1. The appropriate amount of each compound to give the final dietary concentration desired was diluted in 10 gm of corn starch by grinding in a mortar and pestle. This mixture was then added to the appropriate amount of ground laboratory chow and mixed with a mechanical mixer. Rats were fed the respective diets for eight weeks; food consumption in each group was measured during the sixth week of treatment. The hexobarbital sleeping time was tested in ten rats of each group eight weeks after onset of exposure to the treated diets. The hexobarbital was dissolved in water, 125 mg/kg was injected intraperitoneally (ip) into each rat, and the time from the loss of the righting reflex to its recovery was measured. The remaining five rats of each group were killed and the livers were removed for microscopic study. The tissue was fixed in phosphate-buffered 10% formaldehyde solution and stained with hematoxylin-eosin for examination under the light microscope. Formaldehyde solution-fixed frozen sections were stained with oil red O to show fat accumulation. Tissue for the electron microscope was fixed in phosphate-buffered 5% glutaraldehyde, postfixed in phosphate-buffered 1% osmium tetraoxide, dehydrated, and embedded in resin (Maraglas). Sections were cut with a glass knife, stained with lead citrate,[11] and examined with an electron microscope (Philips 300).

In a preliminary study, five adult Sherman strain rats were fed a diet containing 500 ppm of technical DDT and 100 ppm of dieldrin for six weeks and their livers were studied with the light and the electron microscopes.

Results

The animals fed 500 ppm of DDT showed a light tremor and those in the preliminary study fed 500 ppm of DDT and 100 ppm of

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produced a 100% mortality to flies at concentrations that produced only slight mortalities when tested alone. Certainly results of this nature are obviously missing in the current study.

In view of previous comments precluding the extrapolation of cell culture data to whole animal effects, it is difficult to make relevant predictions concerning the effect on man of pesticide-chemical interactions from our work with human cells in tissue culture. What can be said, however, is that using the cell strains described and under the conditions specified in this report, the interaction at the level of synthesis of cellular macromolecules between the pesticides, DDT and parathion, and the other commonly occurring biologically active chemicals, such as aspirin, caffeine, and the industrial PCB plasticizer, is dependent upon the cell line and the additive used and that in most cases no significant interaction appeared to occur. It can be concluded further that in this particular study the responses of human cells of a malignant and a nonmalignant origin to various biologically active environmental chemicals do not differ significantly, although a trend toward nonmalignant cells being less sensitive can be observed.

This study was taken from a dissertation submitted by Dr. Litterst in May 1970 in partial fulfillment of the requirements for the PhD degree. It has been approved for publication by the director of the Wisconsin Agricultural Experiment Station.

This study was supported in part by the State of Wisconsin and by grant FD-00258 from the Public Health Service. It is a contribution from the Wisconsin Agricultural Experiment Station which acted as a collaborator under North Central Regional Cooperative Research Project 85. "Reduction of Hazards Associated With the Presence of Residues of Insecticidal Chemicals in the Environment."

Gerald C. Mueller, PhD, University of Wisconsin Medical School, in addition to furnishing the HeLa cell stock, also supplied the tritiated thymidine, uridine, and leucine; Robert I. DeMars, PhD, University of Wisconsin Genetics Department, supplied the diploid fibroblast cells; and Richard F. Thomas, PhD, US Department of Agriculture, Beltsville, Md, furnished samples of the polychlorinated biphenyl industrial plasticizer as Aroclor 1254.

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Table 1.—*Liver Weights and Sleeping Time of Rats Treated With DDT and Dieldrin*

Treatment	Parameter	Body Weight Gain, gm	Liver Weight, % of Body Weight	Sleeping Time, min
None (controls)	N	15	15	7
	Range	62 — 139	2.41 — 3.06	46 — 98
	Mean ± SE	93 ± 5.7	2.81 ± 0.06	67.1 ± 5.9
Dieldrin, 50 ppm (2.64 mg/kg/day)	N	15	15	9
	Range	36 — 103	2.51 — 3.70	10 — 58
	Mean ± SE	77.0 ± 4.6	3.25 ± 0.08*	39.3 ± 4.3§
Dieldrin, 100 ppm (5.33 mg/kg/day)	N	15	15	9
	Range	34 — 108	3.26 — 4.17	24 — 46
	Mean ± SE	73.0 ± 4.9	3.74 ± 0.07*†	35.7 ± 2.2§
DDT, 250 ppm (12.93 mg/kg/day)	N	15	15	9
	Range	55 — 111	3.09 — 4.35	5 — 47
	Mean ± SE	85.0 ± 4.6	3.47 ± 0.10*	31.6 ± 4.5§
DDT, 500 ppm (26.5 mg/kg/day)	N	15	15	9
	Range	13 — 111	3.07 — 4.05	15 — 62
	Mean ± SE	66.0 ± 6.9	3.60 ± 0.08*†	30.4 ± 4.5§
DDT, 250 ppm (13.3 mg/kg/day), + dieldrin, 50 ppm (2.66 mg/kg/day)	N	15	15	8
	Range	24 — 116	3.49 — 5.55	20 — 40
	Mean ± SE	74.0 ± 6.6	4.27 ± 0.11*‡	32.5 ± 3.0§
DDT, 500 ppm (25.3 mg/kg/day), + dieldrin, 100 ppm (5.06 mg/kg/day)	N	5		
	Range	5 — 32		
	Mean ± SE	20.0 ± 5.9	...	...

* Significantly heavier than the livers of control rats ($P < 0.05$).
† Significantly heavier than the livers of rats fed 50 ppm dieldrin ($P < 0.005$).
‡ Significantly heavier than the livers of rats fed DDT or dieldrin alone ($P < 0.01$).
§ Sleeping time significantly shorter than that of the controls ($P < 0.05$).

dieldrin had a moderate to severe tremor (Table 1). This last group also gained less weight than the others. The food consumption of all treated animals did not differ from that of the controls.

The sleeping time (Table 1) of all treated groups was significantly reduced from that of the controls ($P < 0.005$) but no significant difference was found among the various treated groups. However, all of the dosage levels used were large, and stimulation of microsomal enzymes and reduction of sleeping time may have been maximal at the lowest dose tested. An occasional animal among the controls as well as in the treated group was not affected at all by hexobarbital. These animals were not included in the statistical evaluation (Table 1).

The mean liver weight and the percent of body weight of the livers (Table 1) were significantly increased in the treated groups ($P < 0.05$). A significant difference in the liver weights was also found between the rats fed 50 ppm of dieldrin and those given 100 ppm of dieldrin or 500 ppm of DDT. The liver weights of the animals fed the combination of 50 ppm of dieldrin and 250 ppm of DDT were significantly higher than those of the animals fed DDT or dieldrin alone. There was no significant difference between the liver weights of rats given 250 or 500 ppm of DDT.

The light and electron microscopic findings are summarized in Table 2. The lipid concentrations in the liver sections varied greatly; however, the variations could not be related to the dietary concentrations of the two chlorinated hydrocarbons. The lowest doses of dieldrin (50 ppm) and DDT (250 ppm) given alone caused enlargement of liver cells around the central veins. The cytoplasm had a smoother appearance and at the 50-ppm dieldrin level, margination was also observed. The dietary concentrations of 100 ppm of dieldrin and 500 ppm of DDT caused cytoplasmic inclusions in a number of cells and the enlargement of the liver cells had spread to a larger portion of the liver lobules. The combination of dieldrin at 50 ppm and DDT at 250 ppm resulted in the enlargement of cells throughout the entire liver lobules and a greater number of liver cells had inclusions in their cytoplasm (Fig 1 and 2). With the combination of dieldrin at 100 ppm and DDT at 500 ppm, inclusions were observed in the cytoplasm of almost all cells and the nuclei of many cells appeared to be exceptionally large. The cytoplasmic

Table 2.—Light and Electron Microscopic Findings in the Livers of Rats Treated With Dieldrin and DDT

Dietary Level	Light Microscopic Findings	Electron Microscopic Findings
Dieldrin, 50 ppm	Enlarged cells around central veins, smooth-looking cytoplasm with margination; lipids, none to a large amount	Moderate increase in SER, atypical mitochondria
Dieldrin, 100 ppm	Enlarged cells around central veins, smooth-looking cytoplasm with margination; inclusions in cytoplasm of 3 of 5 livers; lipids, slight to moderate	Moderate increase in SER with swelling in 2 livers; atypical mitochondria
DDT, 250 ppm	Cells around central veins slightly enlarged; smooth-looking cytoplasm; lipids, mild to moderate	Mild to moderate increase in SER, occasional large myelin figures; atypical mitochondria seen in 1 of 5 animals
DDT, 500 ppm	Enlarged cells except in the periphery of the lobules; moderate number of vacuolated cells with inclusions in 2 of 5 rats; margination; lipids, moderate in 2 of 5	Less glycogen in 2 of 5 livers; marked increase in SER, swollen in some areas; occasional myelin figures; occasional atypical mitochondria in 3 of 5 livers
Dieldrin, 50 ppm, + DDT, 250 ppm	All cells enlarged, smooth cytoplasm, margination, inclusions moderate to many; lipids, mild to moderate	Moderate to marked increase in SER, swollen in some areas; less glycogen, slight to moderate number of atypical mitochondria
Dieldrin, 100 ppm, + DDT, 500 ppm	All cells enlarged, many cells with exceptionally large nuclei; margination in cytoplasm and inclusions in almost all cells; lipids, not studied	Marked increase in SER, which is swollen; indistinct cell borders; atypical mitochondria; vacuolated areas in cytoplasm surrounded by layers of dense lamellated material; occasional large vacuoles in cytoplasm containing granular material
None	Liver cells normal; lipids, none to moderate	Occasional small myelin figures

inclusions have been described previously.[2,4,7] They differ in appearance from acidophil bodies which are observed, for instance, in viral hepatitis or from Councilman bodies observed in yellow fever. They are usually not homogeneous but are composed of concentric rings of acidophil cytoplasm which surround one or more vacuoles, and they are observed within the cytoplasm of the cells.

On examination of liver sections with the electron microscope (Fig 4 to 8), a number of changes were observed in the cells of the treated animals. An increase in smooth endoplasmic reticulum (Fig 4) was noted in all treated rats and was most pronounced in the rats fed DDT at 500 ppm and in rats fed the combinations. A widening (swelling) of the vesicles of the smooth endoplasmic reticulum was observed in the livers of some of these rats (Table 2).

Many of the livers of the treated animals showed morphological changes of some of the mitochondria (Fig 5 to 6). These changes consisted of an increase in the number of cristae, a double outer membrane or loss of a portion of the outer membrane, and confluence of two mitochondria. The effect on the mitochondria was more pronounced in the livers of rats fed dieldrin or the combinations. Mitochondrial ring-like formations were also observed (Fig 6).

The glycogen appeared to be decreased in the livers of rats fed DDT at 500 ppm and in those of the rats fed the combinations. The number of free ribosomes varied a great deal and did not seem to be dose-dependent. The liver cells of all rats, including the controls, contained lipid vacuoles which varied greatly in number and size. Some of these vacuoles contained several layers of dark membranes in their outer aspects. In addition, myelin figures were observed in many of the livers of treated rats and occasionally also in the controls. Quantitative differences of these various cytoplasmic components are difficult to assess, since only very small portions of the liver are examined under the electron microscope.

The morphological appearance of the liver cells of rats fed the combination of DDT at 250 ppm and dieldrin at 50 ppm differed from that of rats fed DDT or dieldrin alone by its more pronounced increase in smooth endoplasmic reticulum. In this group, the smooth endoplasmic reticulum of all livers examined showed dilatation "swelling" of the vesicles in some areas of the cell, usually in the periphery. The livers of the rats fed the combination of 500 ppm of DDT and

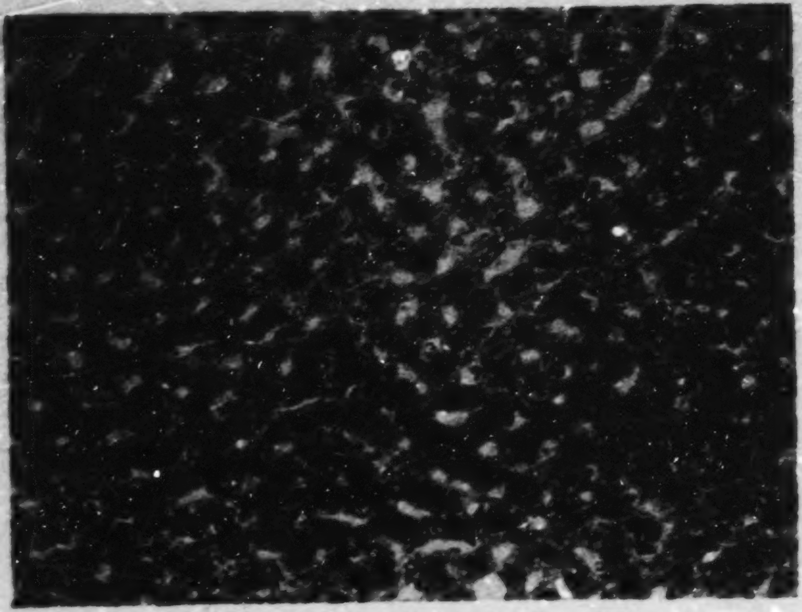
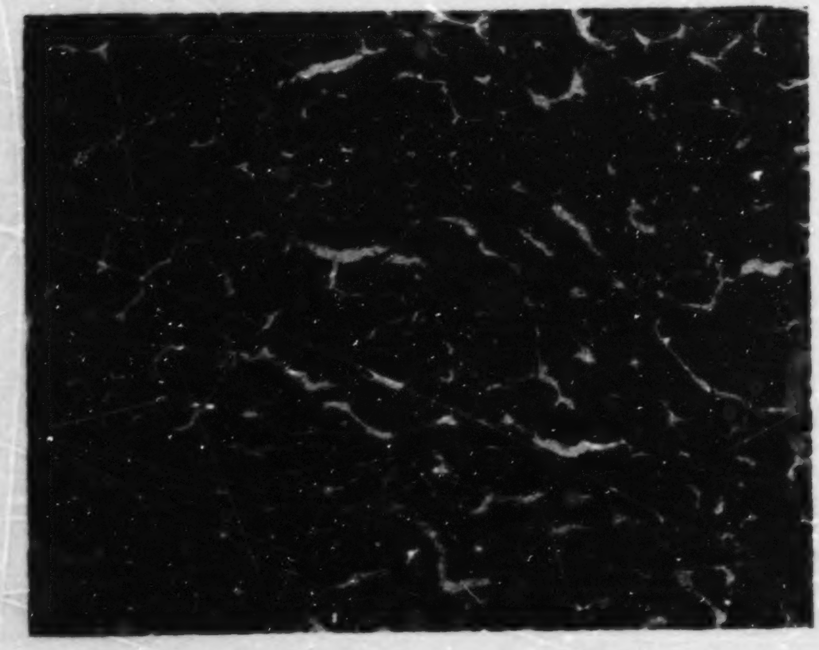


Fig 1.—Normal rat liver (hematoxylin-eosin, × 250).

Fig 2.—Section of liver from rat fed 50 ppm dieldrin and 250 ppm DDT. Note enlargement of liver cells, margination, and cytoplasmic inclusions (arrows) (hematoxylin-eosin, × 250).



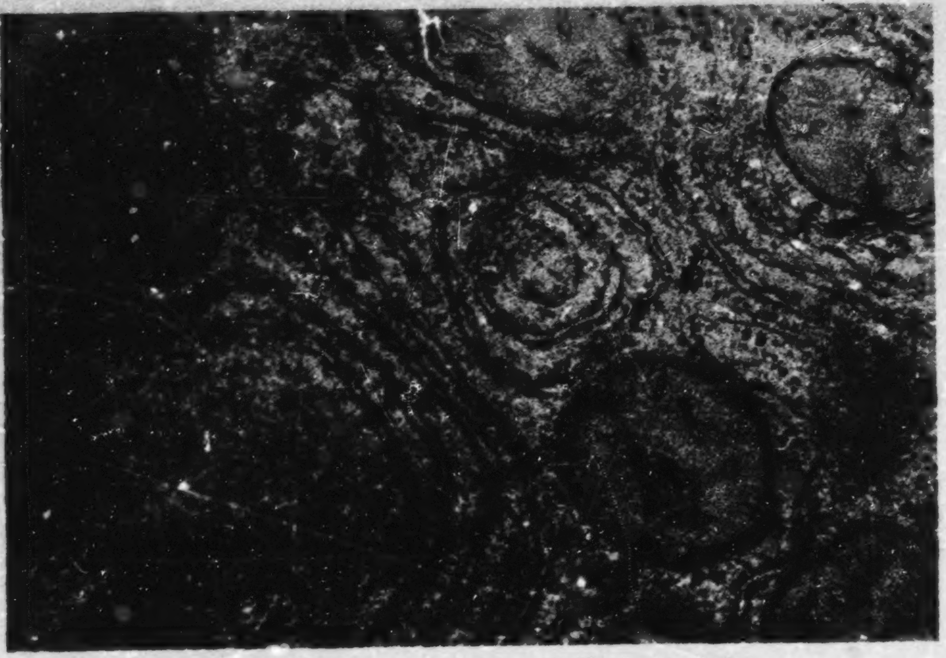
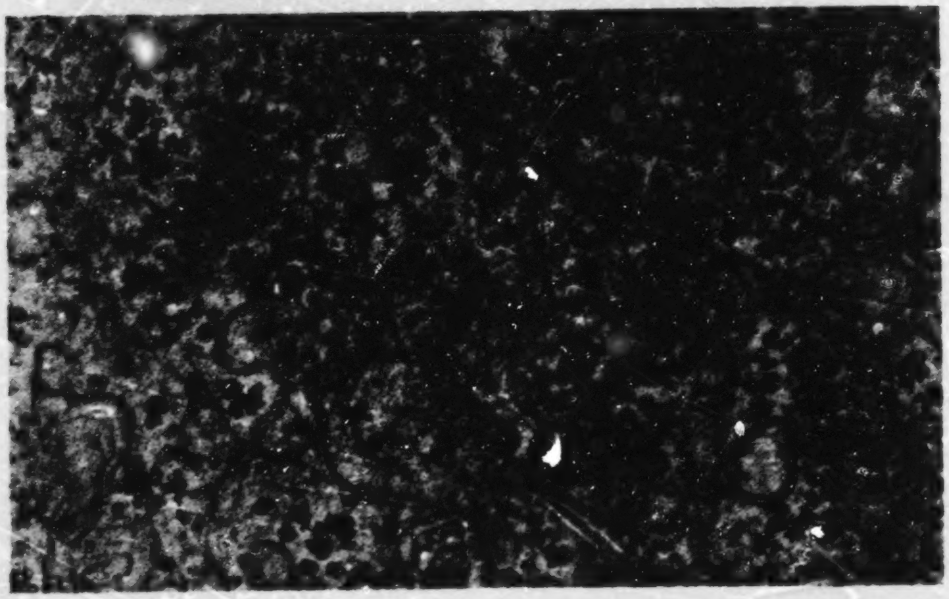


Fig 3.—Segment of a normal rat liver cell. M indicates mitochondria; and RER, rough endoplasmic reticulum (lead citrate, × 42,000).

Fig 4.—Portion of liver cell from a treated rat illustrating increase in smooth endoplasmic reticulum which appears swollen (lead citrate, × 80,000).



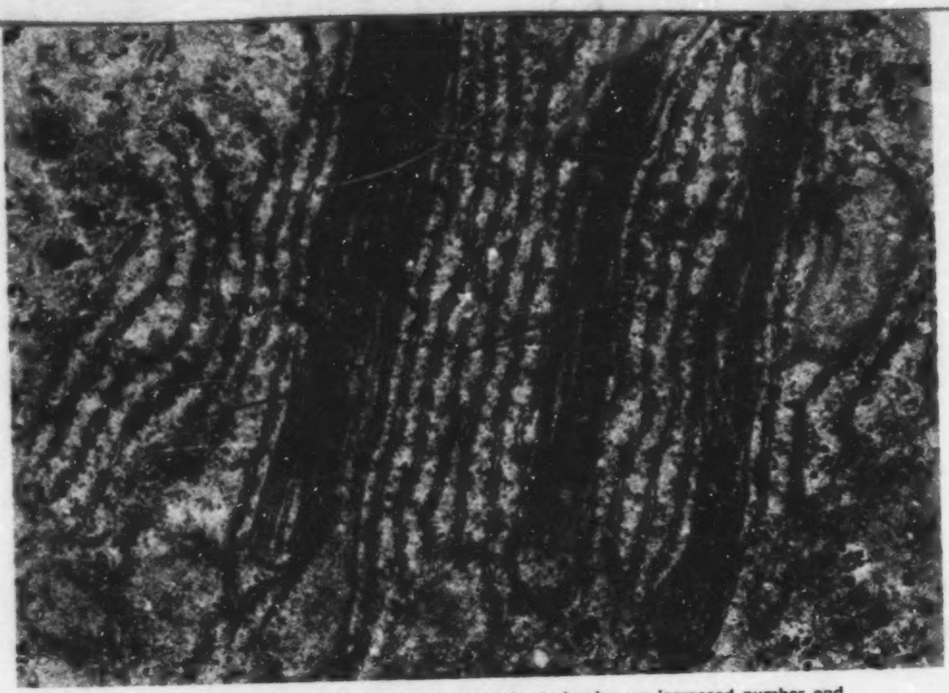
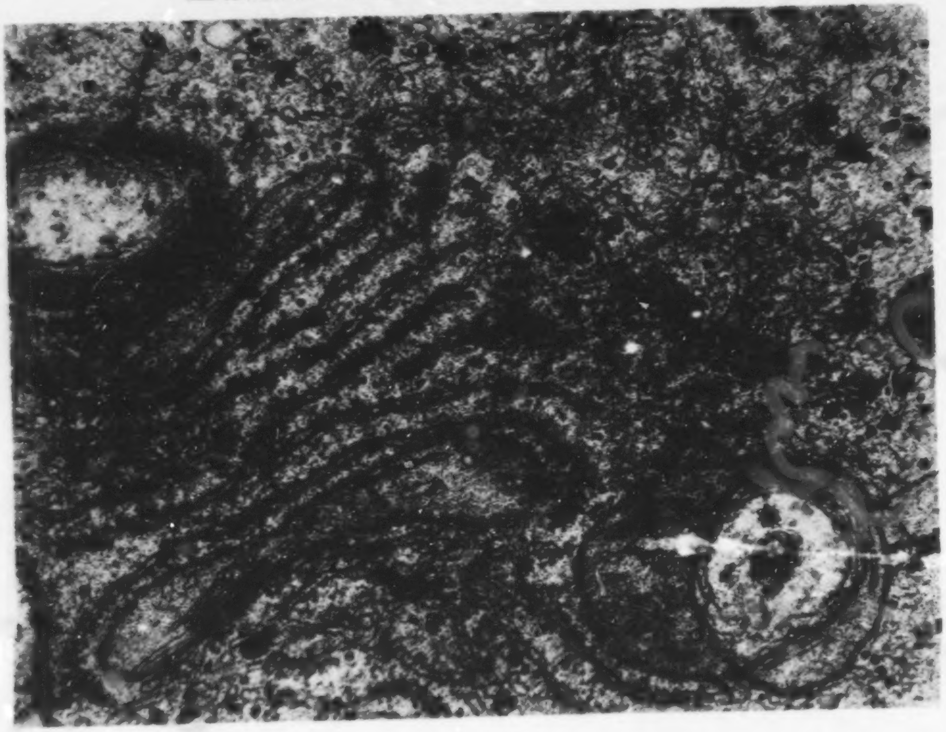


Fig 5.—Portion of liver cell from a treated rat showing an increased number and parallel arrangement of cristae within mitochondria (arrows) (lead citrate, × 64,000).

Fig 6.—Section of liver cell from a treated rat showing ring-like formations of mitochondria (arrows) (lead citrate, × 38,000).



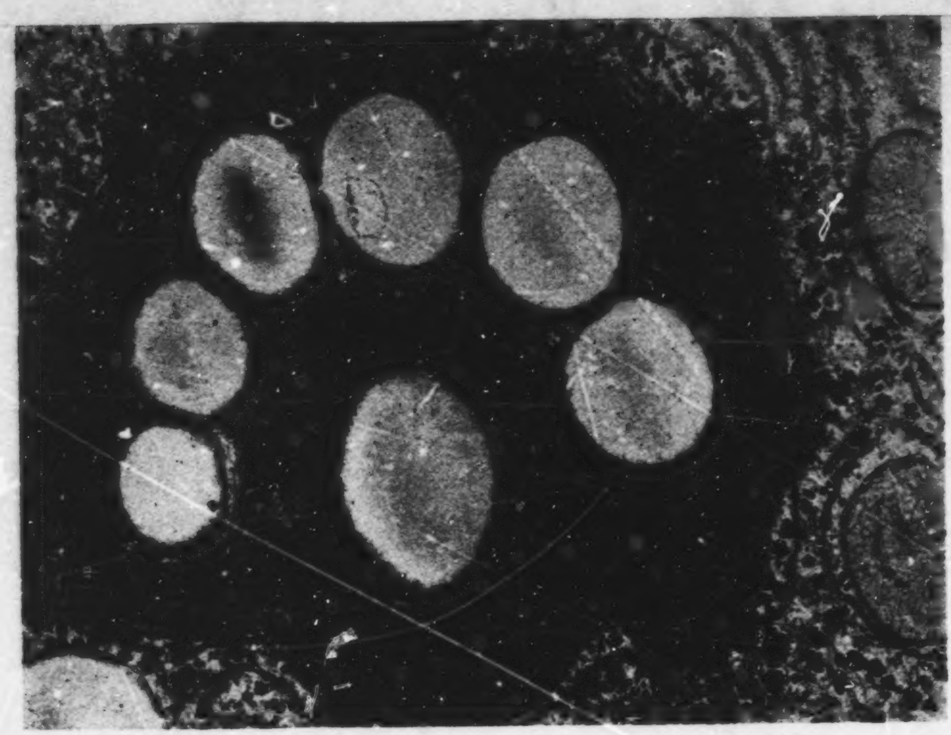
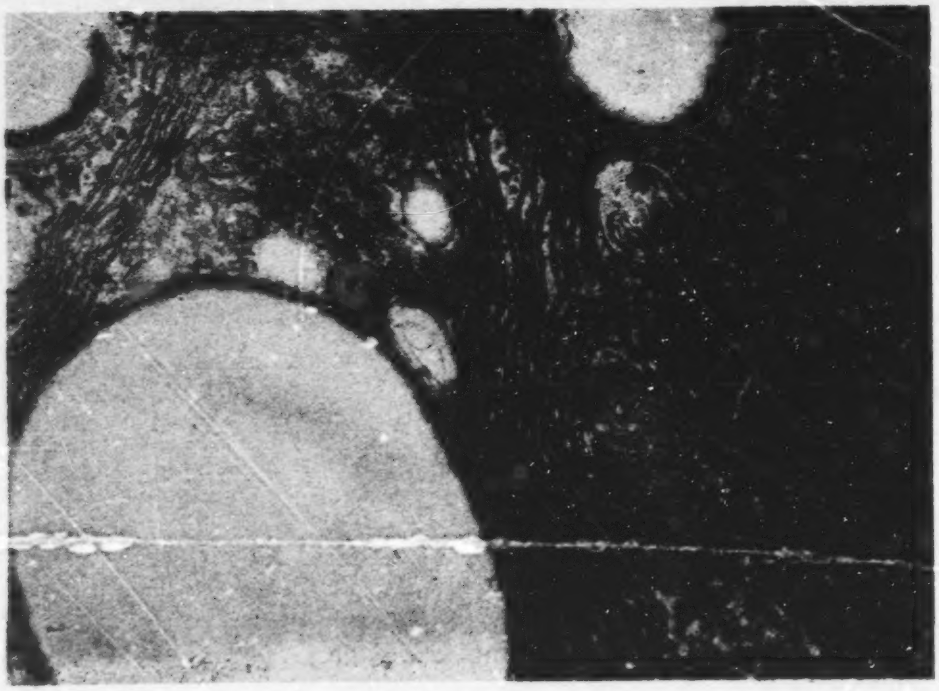


Fig 7.—Concentrically arranged membranes in the cytoplasm of a liver cell from a treated rat. The membranes surround lipid-containing vacuoles (lead citrate, × 24,000).

Fig 8.—Concentrically arranged membranes and an area of finely granular structureless material in the liver of a treated rat (lead citrate, × 27,000).



100 ppm of dieldrin also showed this effect on the smooth endoplasmic reticulum. In addition, these liver cells had indistinct cell borders and vacuolated areas in the cytoplasm which were surrounded by layers of concentrically arranged membranes (Fig 7). Occasionally large areas were observed which contained a granular material but no other cell organelles (Fig 8). The granular material may represent tangentially sectioned layers of concentrically arranged membranes.

Comment

The results of this study show that the hexobarbital sleeping time is reduced in rats pretreated for eight weeks with DDT or dieldrin. The reduction in sleeping time was similar in rats given different dosages of DDT or dieldrin or the combination of the two. However, the liver weights were higher in the animals given the combination of DDT and dieldrin. The livers of the rats given dieldrin at 100 ppm also weighed more than those given dieldrin at only 50 ppm. The increase in liver weight was associated with an increase in smooth endoplasmic reticulum demonstrated with the electron microscope. At present it is widely accepted that the increase in smooth endoplasmic reticulum corresponds to an increase in microsomal enzyme activity of the livers.[7,12-14] However, Hutterer et al[8] found that endoplasmic reticulum can become hypoactive. They gave rats daily small ip doses of dieldrin for 28 days and then increased the dose of dieldrin. The liver weight and the microsomal protein continued to increase but the activity of the drug handling enzymes decreased progressively. The point at which an increase in smooth endoplasmic reticulum is still noted while the microsomal enzyme activity remains stationary or declines will probably show a great species, sex, age, and time-dose related variation, when microsomal enzymes are evaluated for their ability to metabolize additional chemicals. This was also emphasized by Gillett and Arscott[15] in a recent article.

The combination of DDT and dieldrin produces a more pronounced increase in smooth endoplasmic reticulum than either compound alone. The combination also causes enlargement of a greater proportion of the hepatocytes, and a greater number of cells with inclusions in their cytoplasm are observed under the light microscope.

Estelle Gray assisted with the statistical evaluation and Mary Anne Dobbs assisted with the electron microscopic studies. Annie Alford prepared the tissue for the light microscopic examination.

Nonproprietary and Trade Names of Drug

Hexobarbital—*Evipal, Sombulex, Somnalert.*

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Chromosomal Aneuploidies and

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Female mice were given a single intravenous injection of alloxan (60 or 80 mg/kg body weight) and caged with normal males when the blood glucose levels rose significantly. The morning when a vaginal plug was found was designated as day 0.5 of pregnancy. Blastocysts were recovered after sacrifice of mothers on day 3.5 of gestation. Cytogenetic examinations of these embryos were made after air-drying of eggs, and the findings were compared with those observed in embryos taken from control groups. A marked decrease in the number of viable embryos recovered from diabetic pregnant mice was observed. Chromosomal analysis revealed a high incidence of polyploidy and aneuploidy in eggs from the diabetic group as compared with those from controls.

THIS research is part of a long-term collaborative investigation of diabetic embryopathy begun about ten years ago by Watanabe and Ingalls.[1] They initiated alloxan-induced diabetes in pregnant mice and showed that the maternal diabetes resulted in a high mortality among both mothers and fetuses. They also demonstrated a high incidence of cleft palate among surviving embryos. The timing of alloxan injection around the tenth day of gestation was a critical feature of the experiment. When the same amount of the drug was injected in late pregnancy, the maternal mortality dropped and cleft palate was no longer seen in the young.

Subsequently, Horii and his co-workers[2] confirmed the diabetogenetic origin of the fetal malformations by preventing their occurrence with adequate insulin treatment of mothers during pregnancy.

In 1968, Endo and Ingalls found that chromosomal anomalies—breaks and gaps, aneuploidies and polyploidies, also occurred in fetuses, particularly malformed fetuses of mothers with alloxan-induced diabetes initiated prior to conception.[3] The present research was designed to study chromosome constitution of cell clusters in embryos so young that they had not yet hatched from the enveloping zona pellucidas flushed from the uteri of diabetic mothers.

Materials and Methods

The animals used in this experiment were unpedigreed mice from a stock colony maintained at Niigata University for about nine years on a diet of commercial food pellets and water ad libitum. Air temperature was controlled between 15 to 25 C in the animal shelter. Alloxan was dissolved in distilled water immediately prior to injection, since it decomposes rapidly on standing. Virgin female mice more than 12 weeks of age and weighing more than 25 gm were given a single intravenous injection of alloxan in doses of 60 or 80 mg per kilogram of body weight. These two groups will be referred to as the 60- and 80-mg groups.

On the third day after the injection of allox-

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TOXICOLOGY AND APPLIED PHARMACOLOGY 17, 679–690 (1970)

Toxicity of Paraquat to Rats and Its Effect on Rat Lungs

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Toxicity of Paraquat to Rats and Its Effect on Rat Lungs. KIMBROUGH, R. D., and GAINES, T. B. (1970). *Toxicol. Appl. Pharmacol.* 17, 679–690. Acute and subacute toxicity studies were conducted with paraquat (1,1′-dimethyl-4,4′-dipyridylium dimethylsulfate) in adult Sherman rats. The single-dose oral LD50 was 100 mg/kg in male rats and 110 mg/kg in females. The 90-dose oral LD50 in female rats was 21 mg/kg, giving a chronicity factor of 5.2. The acute dermal LD50 was 80 mg/kg in male rats and 90 mg/kg in females. Studies of lung tissue with the electron microscope after a single oral dose indicate that the first discernible changes are pulmonary edema, swelling of the epithelium, an increase in collagen, and an effect on the ribosomes of the membranous pneumocytes. Light microscopic studies of lungs affected by paraquat after po and iv administration or local instillation showed intra-alveolar hemorrhage, edema, extensive fibrosis, and changes in the epithelium. Local instillation of 0.05 mg/kg of paraquat into lung and 0.8 mg/kg into skeletal muscle caused local fibrosis.

The dipyridylium compound paraquat (1,1′-dimethyl-4,4′-dipyridylium dimethylsulfate) is widely used as a weed killer (Thomson, 1967). In their studies on its toxicity in rats, mice, dogs, and rabbits, Clark *et al.* (1966) found that paraquat had a specific effect on the lungs of these animals. The intake of paraquat was followed by fibroblastic and epithelial proliferation, leading to complete consolidation. Several cases of human poisoning have also been reported (Bullivant, 1966; Almog and Tal, 1967; Campbell, 1968; Fennelly *et al.*, 1968; Matthew *et al.*, 1968; McKean, 1968; Oreopoulos *et al.*, 1968). The lungs were similarly affected in these cases, and respiratory failure was the cause of death in most instances. In acute or chronic poisoning with paraquat, the lung lesions may not become apparent until some time after dosing. Daniel and Gage (1966) reported that following a single oral dose most of the compound is excreted in the urine and feces within about 2 days. Because of its rapid excretion and the delayed effect on the lungs, Clark *et al.* (1966) assumed that paraquat initiates irreversible changes in the lungs that do not become apparent until later. Manktelow (1967) found that the pulmonary surfactant was severely reduced or absent in mice that had been given paraquat intravenously. Recently Calderbank (1968), Akhavein and Linscott (1968), and Conning *et al.* (1969) reviewed various actions, properties, and effects of dipyridylium herbicides.

This paper reports the results of our studies of the toxicity of paraquat and its local effect on the lungs of Sherman rats.

METHODS

Adult Sherman rats 3–6 months old were used. They were caged individually and weighed at weekly intervals. The paraquat[1] used was a formulation of the concentrate containing 2 lbs of the paraquat cation per gallon of water. The concentrate was diluted in water in appropriate concentrations for oral, dermal, or intravenous dosing and for instillation into the lungs and skeletal muscle. Dosage or dietary levels were calculated on the basis of the paraquat cation content.

Lung tissue for light microscopic study was fixed in buffered 10% formalin and stained with hematoxylin and eosin. Lung tissue for electron microscopic study was obtained immediately after decapitation of the animals. The tissue was cut into 1–2 mm pieces and fixed in chilled 0.1 M phosphate-buffered 5% glutaraldehyde, postfixed in chilled buffered 1% osmium tetraoxide, embedded in Maraglas following dehydration, and usually stained with lead citrate and uranyl acetate. Sections were cut with glass knives and examined with the Philips EM-300 electron microscope.

Basic toxicity studies. The 1-dose oral LD50 values were determined in 50 male rats weighing 290–595 g and in 50 females weighing 208–290 g. The acute dermal LD50 values were determined in 50 male rats weighing 296–538 g and in 40 females weighing 216–290 g. The paraquat concentrations in water were varied, and the volumes given to the rats were held constant at 0.005 ml/g of body weight by stomach tube and 0.0016 ml/g by dermal application. Otherwise, the methods of conducting the LD50 studies were the same as those described by Gaines (1960). The rats were held for observation for a minimum of 15 days after treatment. Autopsies were not performed on the rats that survived.

The 90-dose LD50 was determined according to methods described by Hayes (1967). Five groups of rats, each consisting of 10 females weighing from 221 to 357 g, were fed paraquat at dietary levels of 300, 400, 500, 600, and 700 ppm, respectively, for 90 days. The diets were prepared by mixing a predetermined amount of paraquat concentrate and an equal amount of 95% ethyl alcohol with 100 g of corn starch, using a spatula. The alcohol served to reduce the surface tension of the paraquat concentrate. The corn starch-paraquat was mixed with 350 g of ground laboratory chow, and then mixed with the proper quantity of ground chow to give the most concentrated diet used. Diets containing lower concentrations of paraquat were prepared by further diluting a portion of the next higher concentration with ground chow. All diets were mixed with a mechanical mixer. The food consumption was recorded daily during weeks 1, 2, 7, and 12 to determine the amount of paraquat consumed in mg/kg/day. To allow time for possible death of the surviving rats, they were held for 2 weeks after termination of exposure. The lungs of all rats that died and of all survivors were examined grossly and microscopically.

The LD50 values were calculated by the method of Litchfield and Wilcoxon (1949).

To provide lung tissue at specified intervals following a single dose, 4 male rats were given 400 mg/kg of paraquat and 2 other males were given 150 mg/kg by stomach tube. At both 1 and 4 hr after dosing, 2 of the rats given 400 mg/kg and 1 control were killed by decapitation. The rats given 150 mg/kg and 1 control were killed 48 hr after dosing. Lung tissue from each rat was examined by the light and the electron microscope.

[1] Supplied through the courtesy of the Chevron Chemical Company.

Dosages of 3.0 and 6.0 mg/kg of paraquat were given to 5 male rats each by tail vein injection to test the effect, if any, of iv paraquat on the lungs. Five control males were given tail vein injections of water only. The paraquat in water was formulated so that it could be administered at a constant volume of 0.0025 ml/g. The rats were anesthetized with ether and killed by cutting the aorta 13 days after treatment, and the lungs were examined grossly and microscopically.

Local instillations. In an effort to establish whether the effect of paraquat on the lung is a direct effect of the compound or the result of some metabolite formed elsewhere in the body, the material was instilled intrabronchially into male rats. The paraquat was dissolved in water in the appropriate concentrations so that the desired dosages could be injected at a constant volume of 0.0003 ml/g of body weight. Thirty drops of India ink per 100 ml of solution were added as a marker. Groups of 3 rats each were given 0.05, 0.1, 0.2, and 0.4 mg/kg of paraquat. Four control rats were given a similar solution without paraquat at 0.0003 ml/g. The dosing procedure for each rat was as follows: While the rat was under ether anesthesia, the trachea was exposed by making a midline incision in the ventral side of the neck, and a small puncture was made in the trachea with a 20-gauge hypodermic needle; a PE-10 polyethylene intramedic tube attached to the dosing syringe was then inserted and passed gently down the trachea; when resistance was felt the formulation was injected slowly. The rats were sacrificed by severing the aorta after ether anesthesia 7 days after dosing and the lungs were examined grossly and microscopically. Lung tissue from the area that contained carbon pigment in the control rats and the rats given 0.4 mg/kg of paraquat was studied for the presence of surfactant, using the procedure described by Manktelow (1967).

Since a local effect of paraquat did occur in the lungs, 0.134% paraquat solution marked with India ink was injected into the skeletal muscles of 10 male rats. With the rats under ether anesthesia, an incision was made in the skin of the upper thigh. The exposed muscle was injected with the solution at the rate of 0.006 ml/g of body weight to provide the rats with 0.8 mg/kg of paraquat. Ten control rats were treated in a similar manner with plain water marked with India ink. Two control rats and 2 treated rats each time were sacrificed after 2 and 4 weeks, and then at monthly intervals. The area of injection, identified by the presence of carbon, was removed and fixed for microscopic study.

RESULTS

Basic Toxicity Studies

The acute oral LD50 for paraquat in rats was 100 mg/kg (19/20 confidence limits, 87–117 mg/kg) in males and 110 mg/kg (90–134 mg/kg) in females. The acute dermal LD50 for paraquat was 80 mg/kg (60–96 mg/kg) in male rats and 90 mg/kg (74–110 mg/kg) in females.

The results of a 1-dose and 90-dose LD50 study for paraquat by the oral route in female rats are shown in Table 1. The chronicity factor (Hayes, 1967) of 5.2 for paraquat indicates that the compound has a moderate cumulative effect in this species. If a marked cumulative effect existed, a higher value would have been obtained, since only a very small portion of the 1-dose LD50 is necessary to kill half the test animals in 90 days. Paraquat has a moderate cumulative effect which is comparable to that of technical DDT (unpublished data).

TABLE 1

ORAL TOXICITY OF PARAQUAT IN ADULT FEMALE RATS

Parameter	1-dose	90-dose
Number of rats tested	50	60
Survival time (days)	3–13	17–88
LD50[a]	110	21
19/20 Confidence limits[a]	90–134	19–23
Lowest dose to kill[a]	75	17
LD1[a]	45	13
Lowest dose causing clinical signs[a]	50	15
Lowest dose tested[a]	25	7.2[b]
Chronicity factor[c]	—	5.2

[a] Expressed as mg/kg for 1-dose test or mg/kg/day for 90-dose test.

[b] Dietary concentration of 125 ppm.

[c] 1-dose LD50 ÷ 90-dose LD50.

Clinical signs of acute and chronic poisoning included diarrhea, wheezing, irregular and rapid breathing, and red stains around the snout. All animals that died showed morphological changes in their lungs. If the rats died within 5–6 days after a single oral dose, pulmonary edema, congestion, and intra-alveolar hemorrhage predominated. In animals that survived a single dose for 10 days or more, fibrosis predominated in the

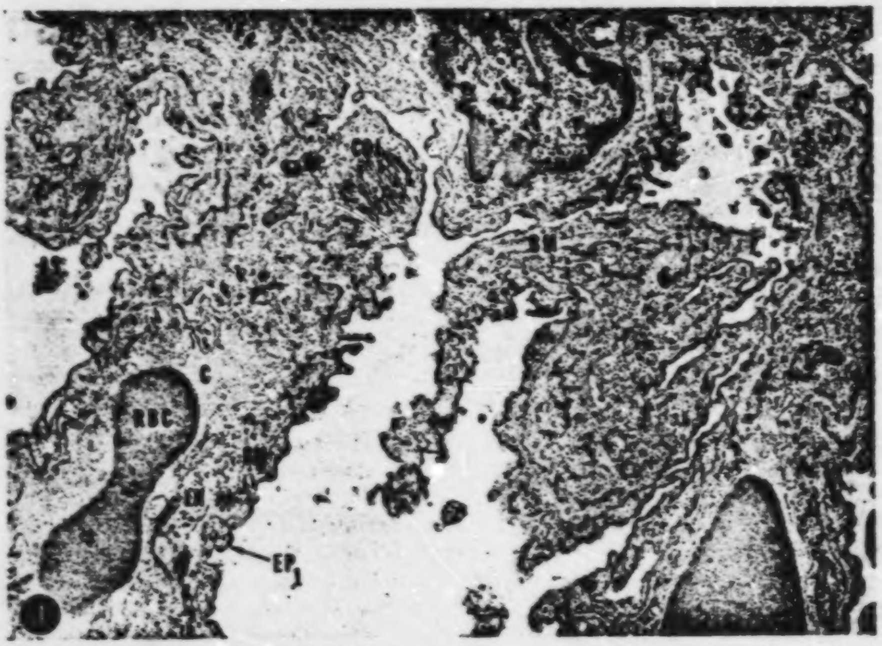

FIG. 1. Normal lung. *C*, capillary; *BM*, basement membrane; *EP*, membranous pneumocyte; *EN*, endothelial cell; *COL*, collagen; *AS*, alveolar surfactant; *RBC*, red blood cell. Uranyl acetate and lead citrate stain. ×8500.

lungs. Occasional multinucleated cells, epithelial proliferation, perivascular lymphocyte infiltration and squamous metaplasia were also observed.

All 33 animals that died in the 90-dose LD50 study had extensive diffuse fibrosis of the lung except for 1 rat which was given 300 ppm of paraquat. The lungs of this animal showed extensive intra-alveolar hemorrhage. Other findings in the lungs, but occurring less frequently than the diffuse fibrosis, were intra-alveolar hemorrhage, multinucleated epithelial cells, proliferation of the epithelium, periarteritis or perivascular lymphocyte infiltration, and a pink-staining homogeneous material lining the alveoli. The lungs of the rats that survived showed circumscribed areas of fibrosis which ranged in size from areas discernible only under the microscope to those measuring 1–2 cm in diameter.

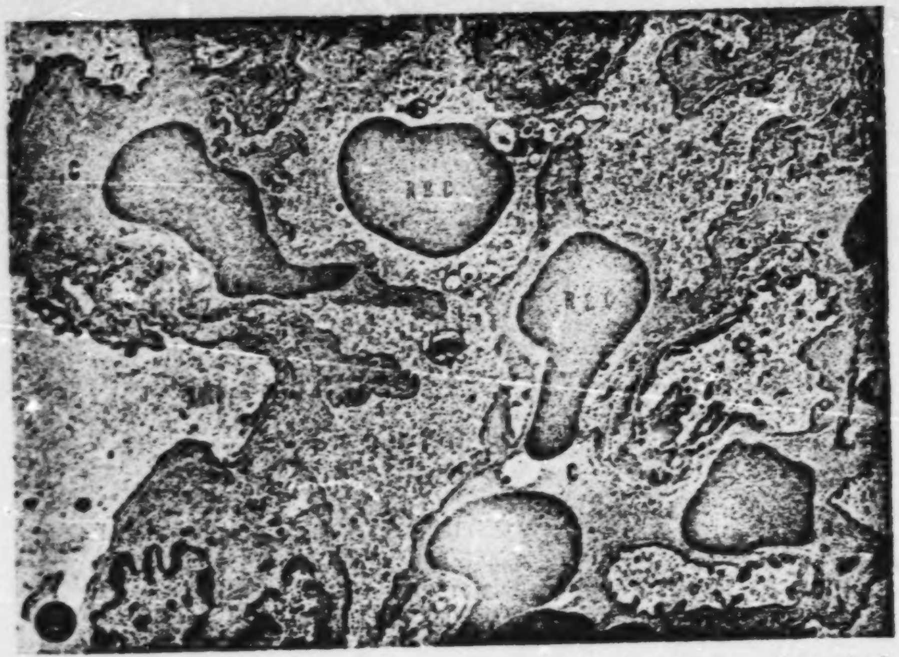

FIG. 2. Lung section from rat 4 hr after paraquat (400 mg/kg, po). Note pulmonary edema and red blood cell within alveolar space. *C*, capillary; *RBC*, red blood cell. Uranyl acetate stain. ×5600.

Lung tissue examined under the electron microscope 1 hr after paraquat administration by stomach tube at 400 mg/kg did not differ from that of the controls (Fig. 1). Four hours after a dose of 400 mg/kg, edema fluid and occasional red blood cells were observed within the alveolar spaces (Fig. 2). The granular pneumocytes were normal (Fig. 3). In some areas swelling of the cytoplasm of the membranous pneumocytes was observed (Fig. 4). Small dark granules normally present within the cytoplasm of membranous pneumocytes became more prominent.

Examination of lung tissue 48 hr after the animals were given paraquat by stomach tube at 150 mg/kg showed an increase in the prominent dark granules within the cytoplasm of the membranous pneumocytes that did not exhibit advanced stages of degeneration. The alveolar lining cells contained large vacuoles within their cytoplasm (Fig. 5). Collagen and elastin were increased, the alveolar lining cells were separated by large spaces from the endothelial cells, and the cells exhibited degenerative change (Fig. 6).

In both control rats and rats given paraquat, a material of laminated or striated structure (Fig. 7) was observed within the alveoli; it probably represents pulmonary surfactant. In the animals killed 4 hr after paraquat administration, the appearance of this material did not differ from that of the control rats. Mendenhall *et al.* (1967) studied the appearance of pulmonary surfactant under the electron microscope after centrifugation. Their material was not processed in the same manner as the lungs in our study, but their photomicrographs also suggest a lamellar structure of alveolar surfactant. This finding is also supported by the studies of Kikkawa *et al.* (1968) and Goldenberg *et al.* (1969).

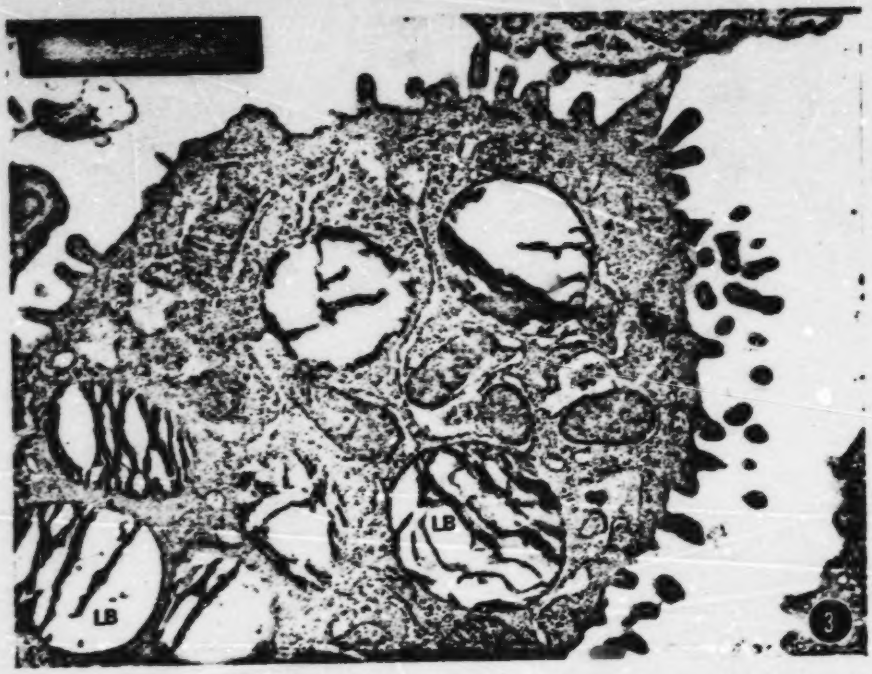

FIG. 3. Lung section from rat 4 hr after paraquat (400 mg/kg, po). Note normal cytoplasm of granular pneumocyte. *LB*, lamellar body. Lead citrate stain. ×17,000.

The light microscopic examination of the lungs studied by the electron microscope 4 and 48 hr after paraquat administration showed intra-alveolar hemorrhage, perivascular lymphocyte infiltration, and prominent alveolar epithelial cells with dark nuclei.

Intravenous injection of paraquat at 6.0 mg/kg in male rats killed 2 of 5 rats tested and produced extensive fibrosis in the lungs of the survivors. The 5 rats given 3.0 mg/kg iv survived; their lungs were normal grossly and microscopically.

Local Instillations

Intrabronchial injections of solutions of paraquat marked with India ink were limited to 1 or 2 lobes of the lung, but not always to the same lobes. The circumscribed areas were easily discernible at autopsy 7 days after injection. The lung tissue in this area showed carbon deposits microscopically. The lowest dosage level of paraquat given, 0.05 mg/kg, produced fibrosis and epithelial proliferation in the areas that contained the carbon pigment (Fig. 8). This amount was 120 times less than the dose necessary to produce lung lesions when the material was injected into the tail vein. The remaining lung tissue not containing carbon pigment was normal. Carbon pigment but no other morphologic changes were observed in lung tissue of controls which had been given carbon pigment in water intrabronchially. Frozen sections of lung tissue from rats that

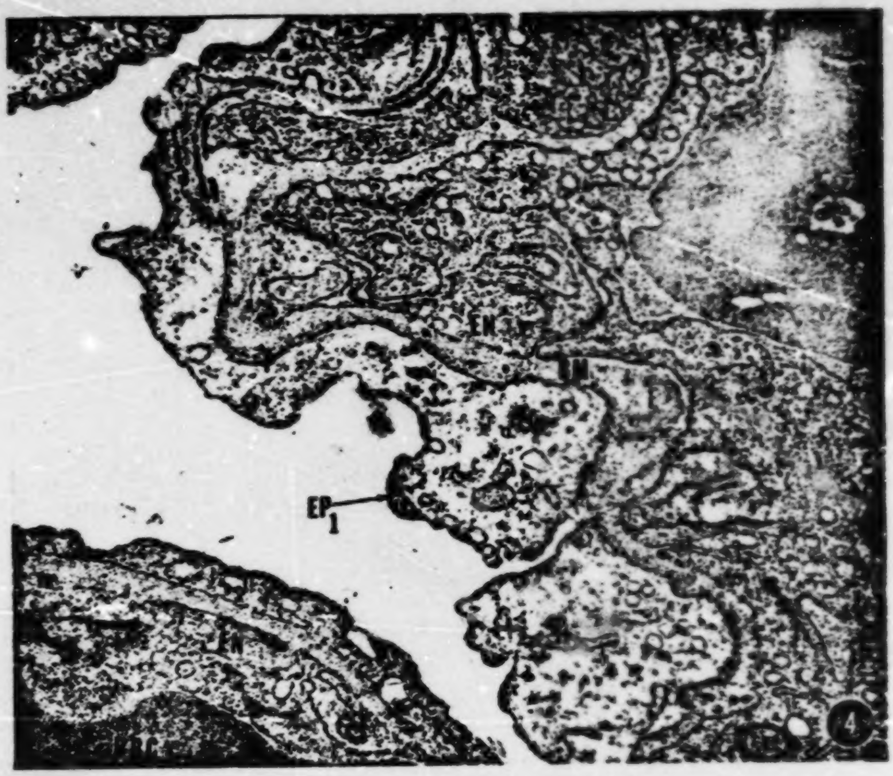

FIG. 4. Lung section from rat 4 hr after paraquat (400 mg/kg, po). Note swelling of the cytoplasm of the membranous pneumocyte. *EP*, membranous pneumocyte; *BM*, basement membrane; *E*, elastin; *EN*, endothelial cell; *RBC*, red blood cell. Lead citrate and uranyl acetate stain. ×25,000.

had been injected with 0.4 mg/kg of paraquat and carbon did not show air bubbles on microscopic examination. Air bubbles were observed in the surrounding lung tissue and also in lung tissue that had only been injected with carbon pigment in water.

A single im injection of paraquat marked with carbon resulted in circumscribed areas of fibrosis in the muscle at the site of the carbon deposit. These areas decreased in size with time and consisted of thin scarlike strands of fibrous tissue 4 and 5 months after injection. Carbon pigment suspended in water was injected into control animals and remained localized; fibrosis was not observed in these areas. The dose used for the im injections was only 7.5 times less than the dose necessary to produce lung lesions by iv injection of paraquat.

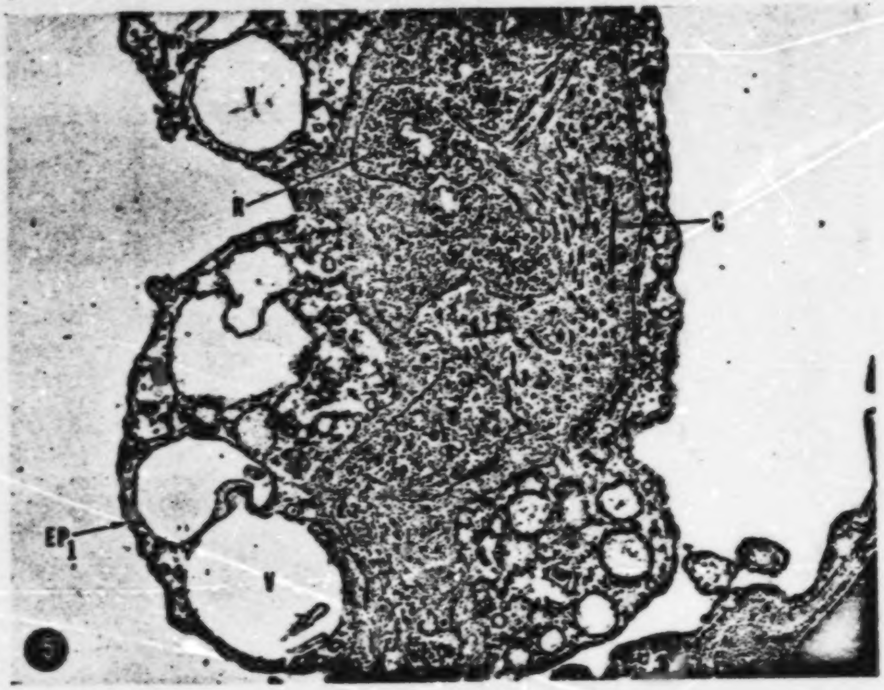

FIG. 5. Lung section from rat 48 hr after paraquat (150 mg/kg, po). Note the large vacuoles within the cytoplasm of the membranous pneumocytes and the prominent dark granules. *EP*, membranous pneumocyte; *V*, vacuoles; *R*, dark prominent granules; *C*, collagen. Lead citrate and uranyl acetate stain. ×22,600.

DISCUSSION

An effect of paraquat on lung tissue is again demonstrated. If a high single dose is given, intra-alveolar hemorrhage occurs and the rat dies in 5–6 days. At a lower dose the rats die later and fibrosis of the lung predominates. Repeated oral doses high enough to cause an effect will, after a period of time, lead to fibrosis of the lung. The chronicity factor, obtained by dividing the 1-dose LD50 by the 90-dose LD50 (Hayes, 1967), is

5.2, a value which is comparable to that of technical DDT (unpublished data). Local single instillations either into the lung or the skeletal muscle also result in fibrosis. The lowest iv dose causing lung fibrosis was 6 mg/kg but a dose 120 times less than that instilled locally into the lung produced fibrosis. This observation shows that the lungs are highly sensitive to paraquat and that the compound has a direct effect on the lungs. Since the lung lesions can be caused by injecting a small amount of paraquat directly into the lungs, they are a result of the compound itself and not of a metabolite formed elsewhere in the body. The fibrosing effect of paraquat is also demonstrated by its effect after injection into skeletal muscle, but here a much higher dose was employed. The areas in the lung showing the fibrosis lose their surfactant (Manktelow, 1967). The effect on the surfactant is not necessarily the primary change produced in the lungs by paraquat, since any area of lung tissue with hemorrhage or fibrosis severe enough to alter the alveolar structure would very likely cause the surfactant to disappear.

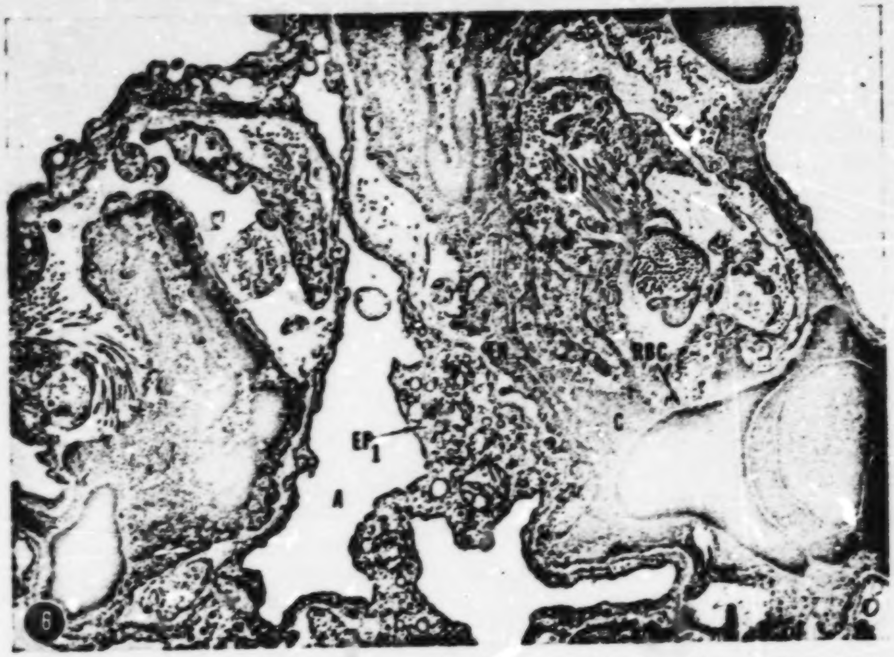

Fig. 6. Lung section from rat 48 hr after paraquat (150 mg/kg, po). Note wide spaces between epithelial and endothelial cells and the swelling of the cytoplasm. *C*, capillary; *A*, alveolus; *RBC*, red blood cell; *COL*, collagen; *EN*, endothelial cell; *EP*, membranous pneumocyte. Lead citrate and uranyl acetate stain. ×8370.

Electron microscopic examination showed an increase in collagen within the intercellular spaces, the formation of elastin, and widening of the intercellular spaces. Granules resembling ribosomes become larger and more prominent in the cytoplasm of membranous pneumocytes. In some areas of the lung the alveolar lining cells show advanced stages of swelling and degeneration 2 days after a single high dose of paraquat.

The findings in this study do not explain why the lung is the primary target organ for paraquat. Kapancy *et al.* (1969) found that breathing pure oxygen at 1 atmosphere of pressure almost completely destroyed alveolar lining cells in monkeys. The destroyed membranous pneumocytes (type 1) were replaced by granular pneumocytes (type 2). Thickening of the air-blood tissue space was caused by an increase in granular pneumocytes and interstitial fibers. The proliferation of the granular pneumocytes was not observed in our study at the dosage levels and time intervals investigated.

Klaus *et al.* (1962) found evidence which supports the hypothesis that the surfactant of the lungs develops during the process of transformation of mitochondria to lamellar bodies in the granular pneumocytes. Macklin (1954) also postulated that the pulmonary surfactant is secreted by the granular pneumocytes. According to Schaefer *et al.* (1964), the lamellar bodies in the granular pneumocytes are responsible for the secretion of alveolar surfactant. These bodies were not affected 4 hr after the administration of paraquat. Likewise, the mitochondria of the granular pneumocytes appeared to be normal 4 hr after an oral dose of paraquat, while ultrastructural changes were observed in the membranous pneumocytes. The lamellated material within the alveoli (Fig. 7), which most likely represents surfactant, was also unaltered. Very early ultrastructural change of the organelles presumably representing or related to pulmonary surfactant could therefore not be demonstrated.

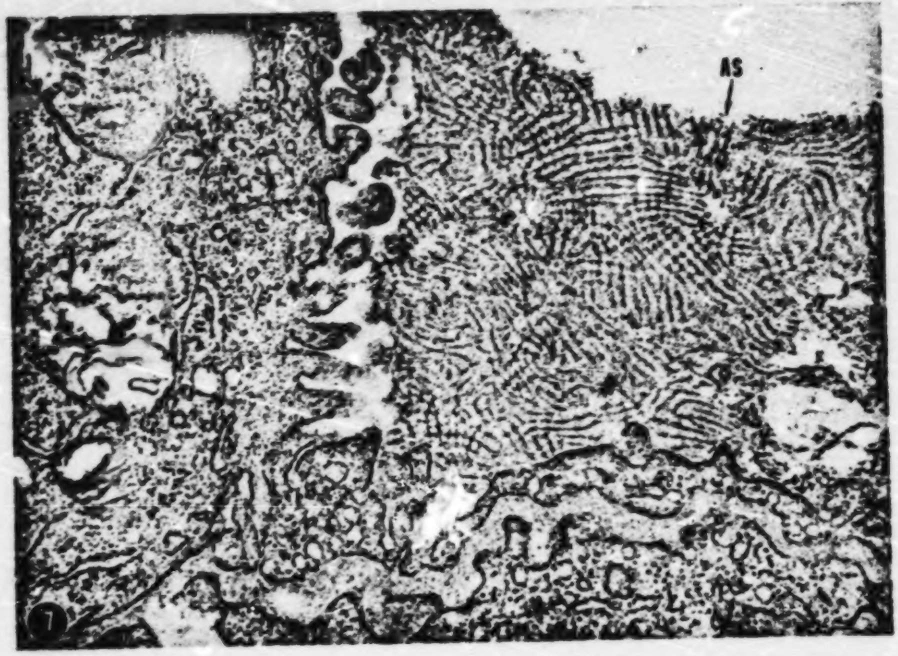

FIG. 7. Lung section illustrating striated structure of alveolar surfactant (*AS*). This material is observed in both normal and paraquat-treated animals. Lead citrate stain. ×15,400.

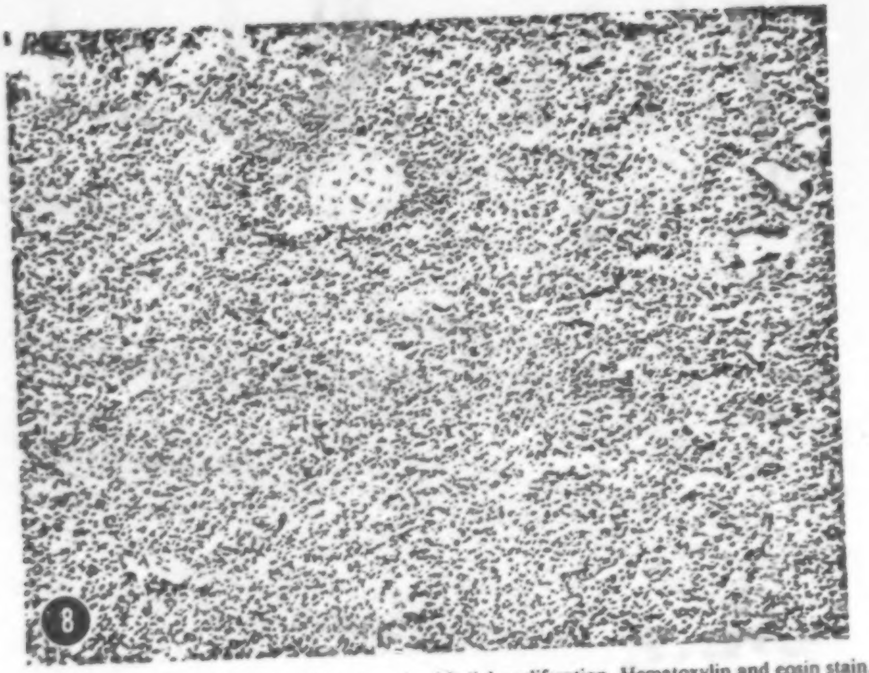

FIG. 8. Lung section illustrating fibrosis and epithelial proliferation. Hematoxylin and eosin stain. ×125.

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PUBLICATION

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PESTICIDES PROGRAM
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Toxicology and Applied Pharmacology, 10(2):406, March, 1967

THE 90-DOSE LD_{50} AND A CHRONICITY FACTOR AS MEASURES OF TOXICITY

W. J. Hayes, Jr.

A 90-dose LD_{50} (or ED_{50}) and a chronicity factor are proposed to help communicate the results of tests involving repeated doses of compounds. To determine the oral 90-dose LD_{50}, groups of animals are fed appropriate dietary levels of a compound for 90 days and then held long enough for any sick survivors to die or recover. Food consumption is recorded and dosages are expressed as milligrams of compound per kilogram of body weight per day. Statistically, the 90-dose LD_{50} is determined in the same way as the 1-dose LD_{50} using logarithms of dosages and percent mortality expressed as probits. A ratio of the 1-dose LD_{50} and 90-dose LD_{50} of a compound is a measure of its cumulative effects and is termed the chronicity factor. The largest one found so far is more than 500 times the smallest found. These factors permit objective comparison of different classes of compounds but whether the smaller distinctions within a class are significant, remains to be learned.

By plotting the logarithm of time in days necessary to kill half of each group of animals against the logarithms of the daily dosages necessary to produce this effect, one may predict the 90-dose LD_{50} of some compounds with acceptable accuracy, but only a much smaller, limiting value for other compounds.

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THE DIAGNOSIS AND TREATMENT OF ORGANOPHOSPHATE INSECTICIDE POISONING

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RICHARD H. GADSDEN, Ph. D.[**]

INTRODUCTION

A survey of South Carolina physicians indicated that about 600 cases of pesticide poisoning occur each year in the state. While the toxic agents were not identified, it is reasonable to assume that most of the poisonings were due to organophosphates with parathion being the most likely offender. With a ban of DDT imminent, an increase in the number of poisonings due to the organophosphates may be anticipated. Prompt diagnosis and treatment is essential to prevent death. If treatment is successful complete recovery can be anticipated.

DIAGNOSIS

A. History

Frequently the history of exposure to pesticides either through the skin or ingestion is obtained. Although parathion is the most commonly used organophosphate, others in wide use are shown in Table 1.

B. Symptoms and Signs

Organophosphates exert their effect primarily by inhibiting the enzyme choliesterase. This results in the accumulation of acetylcholine which causes symptoms of excessive stimulation of the parasympathetic nervous system, central nervous system effects and effects on the myoneural junction. The symptoms listed in Table 2 have all been observed, but all may not be present. The route of exposure does not necessarily correlate with symptoms observed. Pupillary constriction is a most valuable sign but is not always present. Parathion is converted to paroxon, which is the active form of the pesticide, in the human body. This may result in delayed onset of symptoms (up to 8 hours after exposure), especially after dermal exposure.

C. Laboratory Studies

Treatment should not be delayed for laboratory confirmation, but blood should be drawn in an EDTA tube for red blood cell and plasma ChE determination. The stomach contents should be saved for subsequent analysis if the material has been swallowed.

Depression of red cell ChE to values of 25 percent of normal plus a reliable exposure history make the diagnosis certain.

Urine may be analyzed for PNP (para nitro phenol), a metabolite of parathion. Parathion is rarely recovered from the blood but can be identified in autopsy specimens.

D. Treatment

If there has been dermal exposure the clothing should be removed and the patient washed as soon as possible. If the toxic material has been ingested, the stomach should be emptied, preferably by emesis, or, if not possible, by gastric lavage. Ventilation of the patient should be carried out. If possible, cyanosis should have disappeared before the beginning of drug treatment to avoid ventricular fibril-

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Table 1

Commonly Used Organophosphorous Pesticides

Azodrin®	DiSyston®	Phorate (Thimet®)
Dasinit®	Guthion® (Azinphosmethyl)	Phosdrin®
DDVP (Vapona®)	Malathion	Phosphamidon (Dimecron®)
Diazinon (Spectrocide®)	Methyl Parathion	Ronnel (Korlan®)
Dibrom® (Naled)	Mocap®	Systox® (Demeton)
Dimethoate (Cygon®)	Parathion	

lation; but if this is not possible, proceed with drug treatment. There are two drugs that may be lifesaving; atropine sulfate in large dosage and PAM (pralidoxime chloride). Of the two, atropine is usually readily available. The problem is to convince the physician to give large enough doses of atropine. For severe cases atropine sulfate should be given in dosage of 2 - 4 mg (1/32 to 1/16 grain) intravenously every 5 - 10 minutes until full atropinization occurs as indicated by dilated pupils, dry flushed skin, and a rising pulse rate. For children a dosage of 0.05 mg/kg should be given every 5 - 10 minutes. The dosage of atropine may appear large, but patients poisoned with a ChE inhibitor can tolerate these very large doses. The most common error is to give insufficient atropine. Full atropinization should be maintained for at least 24 hours in milder cases of poisoning and at least 48 hours in severe cases. A recent case of a 15 months old child in MUSC Hospital required 52 mg atropine over a five day period. In adults more than 50 mg has been given in a 24 hour period. While atropine blocks the effect of the physiologic response to parasympathetic stimulation, it does nothing to restore the ChE values to normal. Frequently, the binding of ChE can be at least partially released by the administration of a specific antidote Pralidoxime Chloride, usually referred to as Protopam or 2-PAM. The dosage for adults is 1 gm given slowly, intravenously, and

Table 2

Symptoms and Signs

Mild to Moderate	**Severe**
Anorexia	Mental confusion
Nausea	Pulmonary edema
Vomiting	Muscular twitching
Abdominal cramps	Convlusions
Diarrhea	Coma
Weakness	
Excessive sweating	
Excessive salivation and tearing	
Headache	
Pupillary constriction	

repeated in one hour if necessary. The dosage for children is 10 - 50 mg/kg. Little benefit can be obtained from further doses after the second dose. The blood cholinesterase values may rise slightly after the administration of PAM, but not to normal values. Return to normal values may require several weeks. If convlusions interfere with respiration, administer sodium thiopental (2.5 percent) solution intravenously with more than the usual care; poisoning by anticholinesterases sensitizes the medullary centers to depression by barbiturates.

Prognosis

When treatment is instituted promptly, complete recovery can be anticipated unless there has been damage due to hypoxia.

SUMMARY

Organophosphate poisoning is a not uncommon occurrence in South Carolina. The diagnosis can usually be made on the basis of history and physical examination. Laboratory studies aid in confirmation. Prompt treatment with massive doses of atropine sulfate and Protopam may be lifesaving. Complete recovery can be anticipated.

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Effect of Organic Phosphorus Compounds and Alkylating Agents on the Rat Fetus

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The effects of parathion, dichlorvos, diazinon, malathion, apholate, and tepa on the rat fetus were studied when the material was given intraperitoneally. At levels producing toxic symptoms in the dams, parathion, dichlorvos, diazinon, apholate, and tepa were found to affect the fetus either by causing malformations, resorptions, or reducing the weight of the fetus and placenta. Malathion at toxic dosage levels neither affected the weight of the fetus nor produced malformations. At dosage levels which were tolerated by the dams, parathion, diazinon, and tepa caused a great number of resorptions. The incidence of resorptions was not higher than that of the controls in rats that had been given dichlorvos, malathion, or apholate.

THE TERATOGENIC effect of the organic phosphorus pesticides, parathion (0, 0-diethyl-0-*p*-nitrophenyl phosphorothioate), dichlorvos (2, 2-dichlorovinyl dimethyl phosphate), diazinon (0, 0-diethyl 0-[2-isopropyl-6-methyl-4-pyrimidinyl] phosphorothioate), and malathion (diethyl mercaptosuccinate, *S*-ester with 0, 0-dimethyl phosphorodithioate), and the alkylating agents, apholate (2, 2, 4, 4, 6, 6-hexahydro-2, 2, 4, 4, 6, 6-hexakis [1-aziridinyl]-1, 3, 5, 2, 4, 6-triazatriphosphorine) and tepa (tris[1-aziridinyl]phosphine oxide), on the rat fetus was studied. A teratogenic effect of another alkylating agent metepa (tris[2-methyl-1-aziridinyl] phosphine oxide) has been established previously by Gaines and Kimbrough[1] and some of the results will be included in this paper for comparison. Furthermore, Young-

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The Toxic and Teratogenic Effect of Several Organic Phosphorus
Three Alkylating Agents Given Intraperitoneally to Rats on the

Compound	Dose (mg/kg)	Lowest Dose to Kill Nonpregnant Rat (mg/kg)	No.* Pregnant Rats	Average Weight Gain† of Dams (gm)	Average No. Fetuses per Litter	Average No. Dead Fetuses per Litter	Average No. Resorptions per No. Pregnant Rats
Control	—	—	4	67.78	10.6	0	0.5
Parathion	3.5	4	5	42.27	5.6	0.2	7.0
Control	—	—	5	75.24	12.6	0	0.8
Parathion	3.0	—	5	41.79§	7.6	0	3.8
Control	—	—	6	71.70	10.8	0	1.0
Dichlorvos	15‖	40	4	63.43	10.3	0	1.0
Control	—	—	4	85.10	12.5	0.3	0.8
Diazinon	200	300	5	2/5 pregnant rats died	2.0	0.3	11.0
Control	—	—	6	82.38	11.8	0.2	0.7
Diazinon	150	—	6	47.53§	9.2	0	2.8
Control	—	—	4	58.40	13.3	2.3	0
Diazinon	100	—	4	61.11	12.5	0	0.5
Control	—	—	6	92.42	12.3	0	0.5
Malathion	900	1200	6	91.10	11.2	0	0.3
Control	—	—	2	90.11	13.0	0.5	1.5
Malathion	600	—	6	75.73	12.0	0	0.5
Control	—	—	3	81.10	13.3	0	0
Apholate	15	20	4	83.51	13.8	0.3	0.5
Control	—	—	3	74.44	13.0	0	0
Apholate	10	00	6	57.97	13.0	0.2	0.2
Control	—	—	5	Not done	12.4	0	1.4
Tepa	10	20	6	Not done	0	0	12.0
Control	—	—	6	85.20	12.2	0	0.8
Tepa	7.5	—	6	61.25	4.3	1.0	9.2
Control	—	—	4	91.83	14.5	0.5	1.0
Tepa	5	—	4	86.83	14.3	1.5	0.5
Control	—	—	5	81.88	12.2	0	1.0
Metepa	30	40	6	65.11	9.0	2.7	6.7

* In some pregnant rats all fetuses were resorbed thus reducing the total number of litter.
† Weight of placenta and fetus was deducted.
‡ Weight of dead fetuses was included.
§ Significantly different from controls, $p < 0.05$.
‖ A dose of 20 mg/kg dichlorvos killed the pregnant rats.

er[2] reported on a dead, malformed lamb, born to an ewe which had received apholate during pregnancy. The effect of combinations of high doses of organic phosphorus compounds and carbaryl on the chick embryo was reported by Ghadiri and his co-workers[3,4] and for organic phosphorus compounds alone by Marliac.[5]

Materials and Methods

Female Sherman strain rats, 3 to 5 months old and weighing 207 to 309 gm were used. The rats were caged individually except during the mating period when males and females were caged in pairs. Laboratory chow and water were given as desired. Prior to the reproduction study, a range-finding test was conducted to establish the acute toxicity for each compound when given intraperitoneally. Apholate and tepa were dissolved in water and the other compounds were suspended in peanut oil and injected intraperitoneally at the rate of 0.005 cc/gm body weight. In this test five dosage levels in geometric progression were given to two females for each compound. The highest nonfatal dosage level was then chosen for the reproduction study, but if this introduced an effect on the fetus, lower dosages were employed in an attempt to establish a dosage that did not affect the fetus.

In the reproduction study 12 female rats were divided into two groups of six rats for each dosage level of each compound according to a table of random numbers. They were pair-mated for six days with healthy adult male rats

Compounds and 11th Day of Pregnancy		
Average Weight of Fetuses (gm)	Average Weight of Placentas (gm)	No. and Type of Malformations per Total No. Fetuses
3.57	0.69	None
2.48§	0.53	1/28 fetus edematous
3.34	0.54	None
2.86§	0.44§	None
3.52	0.50	None
3.61	0.54	3/41 omphaloceles
3.62	0.49	None
1.52§	0.23§	1/6 hydrocephalus; 1/6 first distal phalanx missing; 1/6 ectromelia
3.14	0.54	None
2.56§	0.40	None
2.89	0.47	None
3.53	0.71	6/50 dilated renal pelvis
3.67	0.57	None
3.31	0.48	1/67 adactylia
3.09	0.43	None
3.41	0.45	None
3.25	0.52	None
3.10	0.40	1/55 ectromelia; 1/55 agenesis of left ribs (7-12)
3.65	0.53	None
2.24§	0.25§	1/78 ectromelia
Not done	Not done	—
Not done	Not done	—
3.64	0.54	None
1.61§	0.21§	None
3.53	0.49	1/58 hydrocephalus
2.54§	0.26§	4/57 edema
3.44	0.53	None
1.25§	0.18§	9/36 ectrodactylia, all paws; 27/36 ectrodactylia, front paws; 17 other anomalies[1]

of about the same age. The females were weighed once a week. Insemination was checked daily by vaginal smears. The day of insemination was counted as day zero. On day 11 after insemination the pregnant rats were given a single intraperitoneal injection of the compound under study. On the 20th day of pregnancy the fetuses were removed by cesarean section under ether anesthesia. The offspring and placentas were weighed separately. The number of resorptions and dead animals was recorded. Half of the offspring were fixed in Bouin's solution for study of serial sections under the dissecting microscope. The sections were cut with a razor blade and were 1 to 2 mm thick. Sections which revealed abnormalities were embedded in paraffin and processed for study under the light microscope. The remaining rats were fixed in ethyl alcohol, cleared with potassium hydroxide, and stained with alizarin red for examination of the bone structures according to the methods of Murphy[6] and Wilson.[7]

Results

The results are given in the Table. Toxic levels of parathion and diazinon caused a high incidence of resorptions and reduced the weight of the fetus and placenta; similar levels of dichlorvos and malathion did not have such an effect.

Parathion, dichlorvos, and diazinon produced some malformations, but the only malformation among controls was one incidence of hydrocephalus. As the dose of the various compounds was decreased, the effect on the offspring was reduced. Results obtained with apholate are not consistent since 10 mg/kg caused a reduction in the weight of the fetus, while 15 mg/kg did not. Tepa, the other alkylating agent studied, caused a very high incidence of resorptions at toxic levels. Even for the lowest dose given, the average weight of the fetuses and placentas was significantly lower than that of the controls. However, malformations were not produced with tepa even though it is very similar to metepa whose teratogenic effect on rats is well established.[1]

The net weight gain of the dams was reduced significantly when 150 mg/kg of diazinon or 3.0 mg/kg of parathion were given intraperitoneally. The higher dose of 3.5 mg/kg of parathion also produced a reduction in weight, but this was not significant. In the case of parathion, metabolite may be responsible for the lower weight of the dams. It is well-known, for instance, that one of its metabolites, dinitrophenol, reduces weight by increasing the rate of metabolism. It also increases the number of stillbirths in rats.[8] The average net weight gain of the dams dosed with other compounds was usually lower, but it cannot be concluded from these findings that any of the various chemicals was responsible for the reduced weight gain since quite a variation was also noted among the various control groups.

Comment

The spontaneous incidence of malformations is very low in our Sherman strain rats (Table). The results obtained show that the organic phosphorus compounds, parathion, dichlorvos, and diazinon, are slightly teratogenic in Sherman strain rats when a single dose of the chemicals is given intraperitoneally on the 11th day of gestation at levels producing at least some symptoms of poisoning in the dams. Malathion at toxic dosage levels neither affects the weight of the fetus nor produces malformations. It is possible that by giving only one dose on the 11th day of pregnancy a more pronounced teratogenic effect was missed which might have been found had the chemicals been given earlier in the gestational period or in repeated doses. The studies were undertaken as a screening method to establish the practical value of obtaining toxicity data on the rat embryo. Feeding studies, rather than intraperitoneal injections, are a more practical approach to establish a possible teratogenic effect for these compounds since human exposure would most likely occur this way. It must be emphasized that only those dosage levels of the organic phosphorus compounds which produced toxic symptoms in the dams affected the fetus.

Decreased body weight of the fetus was found to be the most sensitive indicator of toxic effect on the fetus. This was true for the organic phosphorus compounds as well as the alkylating agents that were given. The weight of the placenta was always reduced when the fetus was smaller.

Metepa which was included in this paper for comparison is highly teratogenic in Sherman strain rats, particularly at high dosage levels. It is interesting that tepa which is chemically closely related to metepa produced only very few malformations even though the fetus weight was reduced and the number of resorptions was much greater than that of the controls at the two higher dosage levels given.

Mrs. Martha Bey, Mrs. Estelle Gory, Mr. Richard L. Moore, and Mr. Ralph E. Linder assisted in these experiments.

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TREE GROWIN'

There's sometimes a good hearty tree growin' right out of the bare rock, out o' some crack that just holds the roots; right on the pitch o' them bare stony hills where you can't seem to see a wheel-barrowful o' good earth in a place, but that tree'll keep a green top in the driest summer. You lay your ear down to the ground an' you'll hear a little stream runnin'. Every such tree has got its own livin' spring; there's folks made to match 'em.—Sarah Orne Jewett: *The Country of the Pointed Firs and Other Stories*, Houghton Mifflin Co., 1896.

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MEASUREMENT OF BLOOD CHOLINESTERASE ACTIVITY IN LABORATORY ANIMALS UTILIZING DIMETHYLBUTYL ACETATE AS A SUBSTRATE[1,2,3]

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SUMMARY ● *Blood cholinesterase activity was measured in the monkey, dog, rat, mouse, and rabbit. The use of the substrate, dimethyl butyl acetate, resulted in expected values for the monkey, dog, and rabbit, but the cholinesterase activity of the rat and mouse was quite elevated. In vivo cholinesterase inhibition resulted in lower values in all animals. Dimethyl butyl acetate was indicated as acceptable substrate for measuring cholinesterase activity except in the mouse and rat, where high levels of non-specific esterases complicated interpretation.*

The sophistication of protocols for the evaluation of subtle effects of anticholinesterase compounds demands methodologies capable of measuring small changes in acetylcholinesterase levels with minimal samples. Additional problems result from the low plasma-specific activity of several rodent species. Many methods are available for acetylcholinesterase determination (1–4); however, most suffer some or all of the disadvantages of low sensitivity, selectivity, or precision, or require specially modified instrumentation.

Described in this report is the application of a highly sensitive and precise method of cholinesterase determination, developed for the analysis of microsamples (20 μl) of human serum, for use with laboratory animals (5). Utilized in the procedure are 3, 3-dimethyl butyl acetate (DMBA), a nonphysiologic acetylcholinesterase substrate (6–8), and a gas chromatographic determinative step. The results of analysis of normal and parathion- and carbaryl-inhibited plasma and red blood cholinesterase of the monkey, rat, mouse, dog, and rabbit are reported and discussed. Results obtained by the pH Stat[4] method (4) are contrasted to those obtained with DMBA.

Materials and Methods

The animals utilized in this study were Crl:CD-1(ICR)BR weanling and mature mice; Hot:(SD)BR weanling and mature rats; NZW, mature rabbits (from a commercial source) mature rhesus (*Macaca mulatta*) monkeys; mature squirrel (*Saimiri sciureus*) monkeys, and mature beagle dogs. Their diets consisted of Purina Rat, Monkey, Rabbit and Dog Chow.[5]

Cholinesterase was determined by the micro-method of Cranmer and Peoples (5). In brief, this method is as follows: 20 μl of packed red cells or plasma were incubated at 37° C in pH 8 buffer for 30 min with DMBA as the substrate. The reaction product, 3,3-dimethyl-1-butanol (DMB), was extracted into carbon disulfide, and a Tracor MT-220 equipped with flame ionization detector and a 6′ x ¼″ Chromosorb 101 column was used to separate and quantitate the reaction product. The method of Nabb and Whitfield (4) was utilized in the determina-

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[4] Sargent-Welch, Skokie, IL.
[5] Ralston-Purina Co, St Louis, MO.

TABLE 1

Cholinesterase values for laboratory animals expressed in micromoles per milliliter per minute

Species	No samples	Plasma cholinesterase mean ±SE (range)	RBC cholinesterase mean ±SE (range)
Squirrel monkey	6	1.1 ± 0.1 (0.8 - 1.4)	4.8 ± 0.2 (4.3 - 5.7)
Rhesus monkey	6	1.7 ± 0.1 (1.4 - 2.1)	6.8 ± 0.6 (4.9 - 8.5)
Dog	6	0.9 ± 0.1 (0.6 - 1.0)	1.0 ± 0.1 (0.8 - 1.2)
Weanling rat	12	4.5 ± 0.4 (2.9 - 6.7)	2.8 ± 0.1 (2.4 - 3.7)
Mature rat	12	5.6 ± 0.4 (3.6 - 9.0)	1.4 ± 0.1 (1.2 - 1.6)
Weanling mouse	11	29.5 ± 0.8 (25.4 - 32.0)	2.0 ± 0.1 (1.5 - 2.9)
Mature mouse	10	19.8 ± 0.9 (17.0 - 24.0)	1.1 ± 0.03 (1.0 - 1.3)
Weanling rabbit	12	1.1 ± 0.3 (0.4 - 2.3)	1.3 ± 0.1 (1.0 - 1.6)
Mature rabbit	11	0.9 ± 0.2 (0.3 - 2.0)	1.0 ± 0.1 (0.7 - 1.2)

tion of cholinesterase with the pH Stat.

In vivo cholinesterase inhibition was produced by administration of approximately ¼ of an LD_{50} of parathion and carbaryl by means of a stomach tube. Plasma and red cell cholinesterase were determined 30 min prior to and after intubation.

RESULTS AND DISCUSSION

Table 1 contains the mean, standard error, and range of plasma and red cell cholinesterase activity of the species studied. Comparison of these data demonstrates an unusually high plasma activity for the rat and the mouse and a considerable change in both RBC and plasma activity with age. Comparison of determinations of cholinesterase by DMBA and acetyicholine was desirable due to the possible species differences in substrate affinity and the knowledge that certain rodents, especially the mouse, have high plasma activities of non-specific esterases. Red cell and plasma cholinesterase values, when DMBA is used, are similar to

TABLE 2

Comparison of GC and pH stat method for cholinesterase expressed in micromoles per milliliter per minute

Species	No samples	Method	Plasma cholinesterase mean ± SE (range)	RBC cholinesterase mean ± SE (range)
Dog	3	pH stat	1.8 ± 0.06 (1.7 - 1.9)	1.7 ± 0.1 (1.5 - 1.8)
	6	GC	0.9 ± 0.1 (0.6 - 1.0)	1.0 ± 0.1 (0.8 - 1.2)
Rabbit	28	pH stat	0.76 ± 0.03 (.48 - 1.2)	2.1 ± 0.18 (0.5 - 2.8)
	11	GC	0.9 ± 0.2 (0.3 - 2.0)	1.0 ± 0.1 (0.7 - 1.2)
Rat	42	pH stat	.62 ± 0.04 (0.3 - 1.2)	1.7 ± 0.07 (1.0 - 2.8)
	12	GC	5.6 ± 0.4 (3.6 - 9.0)	1.4 ± 0.1 (1.2 - 1.6)
Man	18	pH stat	4.9 ± 0.02 (3.6 - 6.8)	13.2 ± 0.31 (11.1 - 16.0)
	25	GC	3.2 ± 0.1 (2.1 - 4.6)	9.8 ± 0.2 (8.2 - 11.8)

TABLE 3

In vivo effect of parathion and carbaryl on laboratory animals

Species	No samples	Mean plasma cholinesterase µM/ml/min		Mean RBC cholinesterase µM/ml/min		% inhibition	
		No inhibition	Inhibition	No inhibition	Inhibition	Plasma	RBC
				Effect of 1/4 LD_{50} Parathion			
Rhesus monkey	6	2.2	0.8	7.0	5.7	64	19
Squirrel monkey	6	1.3	0.3	4.3	2.8	38	34
Weanling rat	6	4.0	1.8	4.3	2.9	54	32
Mature rat	6	16.8	1.0	3.0	1.9	86	37
Weanling mouse	6	16.4	13.9	1.9	1.5	15	21
Mature mouse	6	18.2	14.4	1.6	1.2	21	24
Rabbit	6	1.5	0.4	1.7	1.0	74	40
				Effect of 1/4 LD_{50} Carbaryl			
Squirrel monkey	6	1.7	1.3	6.4	5.6	24	12
Mature rat	6	6.5	5.3	2.4	1.8	18	25
Mature mouse	6	19.8	11.8	1.6	0.9	40	46

pH Stat values except in the plasma of the rat and mouse (Table 2.) The elevated plasma activity with DMBA for these rodents is much higher than values obtained using a choline substrate, which suggests the presence of large activities of non-choline esterase in mouse and rat plasma.

The *in vivo* effect of parathion and carbaryl on laboratory animals was demonstrated using DMBA (Table 3). The results support the conclusion that DMBA is an acceptable cholinesterase substrate for the dog, rabbit, and monkey, but not for the plasma of the mouse, and possibly the rat, where high levels of non-specific plasma esterases complicate interpretation.

The gas chromatographic method for determining cholinesterase is a valuable research tool. The advantages are: the small sample (20 µl), stability in CS_2 when refrigerated, and the wide dynamic range of determination (0.3–32 µM/ml/min). This technic should not be applied to the analysis of mouse plasma, and care should be exercised when the rat is the species investigated.

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